LOCAL EFFECTS OF LINOORBITIDES AND ENTEROLACTONE ON INTESTINAL EPITHELIAL FUNCTIONS

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ABSTRACT

The use of natural products is becoming increasingly popular worldwide. Flaxseed is a source of natural products that are rich with bioactives and have been reported to modulate inflammation, blood pressure, glucose levels and lipid profiles in both humans and animals. Currently, several known bioactives including fibers, lignans, alpha-linolenic acid, and linoorbitides (LOBs) are believed to impart those pharmacological activities. Previous studies have focused on the ability of flaxseed bioactives to provide their pharmacological action in the systemic circulation. However, our increasing understanding of their pharmacokinetics and chemical structures, suggests enhanced abundance of LOBs and lignans at the level of the intestinal epithelium due to the potential lack of permeation of LOBs, and susceptibility of lignans to enterohepatic circulation. Our aim in this work is to evaluate the effects of the lignan enterolactone (ENL) and LOBs on intestinal epithelium function and how it might influence associated pathologies like inflammatory bowel disease (IBD) and hypercholesterolemia.

We first assessed LOBs and ENL effects on barrier integrity (a pathological hallmark of IBD) *in vitro* using a monoculture transwell system, and the potential mechanisms involved in maintenance of the barrier. Next, we examined their effect in a coculture system that more closely simulates the intact intestinal mucosa. These studies triggered the evaluation of pyruvate kinase M-2 (PKM2) as a possible biomarker of IBD. Lastly, we evaluated the effect of lignans on cholesterol metabolism in a human intestinal epithelial cell line (Caco-2). Our barrier integrity assessments of LOBs and ENL in the monoculture system identified differential effects of LOBs and ENL, and both showed increased protein levels of anti-inflammatory peroxisome proliferator-activated receptor gamma (PPAR-γ) and tight junction protein zonula, occludin (ZO-1). Further, in newly diagnosed IBD patients, PKM2 was indicative of disease presence with less variation than other biomarkers like fecal calprotectin and fecal lactoferrin. Lastly, altered cholesterol trafficking was observed in Caco-2 cells, an effect best attributed to the glucuronic acid conjugated form of enterolactone.

These studies confirmed the importance of the flaxseed bioactives, ENL and LOBs in maintaining barrier integrity and modulation of cholesterol metabolism. More importantly, and due to their increased abundance at the level of the intestinal epithelium, those effects are crucially important in IBD and hypercholesterolemia. However, these effects remain to be evaluated in an animal models of colitis and dyslipidemia.

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"In the Name of Allâh (God), the Most Gracious, the Most Merciful"

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Dedicated to

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My brother Amjed, the role model,

My brother Alaa, the challenge creator,

My sister Ala`a, the sound of wisdom,

My little Noor, who brought smiles, happiness and joy (I love you),

&

My dear wife Majd, for your support, patience, constant motivation and being the beauty in my life,

Table of Contents

List of Figures

List of Tables

List of Abbreviations

AAPH: 2,2'-Azobis(2-amidinopropane) dihydrochloride

ABC: ATP-Binding Cassette

ALA: Alpha-linolenic Acid

APCs: Antigen Presenting Cells

ATG16L1: Autophagy Related 16 Like 1

Caco-2: Colon Adenocarcinoma Cell line

CAT: Catalase

CD: Crohn`s Disease.

CDAI: Crohn`s Disease Activity Index

CRP: C-Reactive Protein

CsA: Cyclosporin A

CVD: Cardiovascular Disease

COX: Cyclooxygenase

DCs: Dendritic Cells

DHA: Docosahexaenoic Acid

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethylsulfoxide

DSS: Dextran Sodium Sulfate

ELISA: Enzyme-Linked Immunosorbent Assay

END: Enterodiol

ENL: Enterolactone

eNOS: endothelial Nitric Oxide Synthase

EPA: Eicosapentaenoic acid

ER: Endoplasmic Reticulum

ESR: Erythrocyte Sedimentation Rate

FDA: Food and Drug Administration

GGT: Gamma Glutamyl Transpeptidase

GIT: Gastrointestinal Tract

HBSS: Hank`s Balanced Salt Solution

HDL-C: High Density Lipoprotein-Cholesterol

HMG-CoA: 2-Hydroxyl-methyl-glutaryl Coenzyme A

IBD: Inflammatory Bowel Disease

IBDQ: Inflammatory Bowel Disease Questionnaire

IEC: Intestinal Epithelial Cells

IGF-I: Insulin-Like Growth Factor I

IL: Interleukin

INF-γ: Interferon Gamma

iNOS: inducible Nitric Oxide Synthase

INSIG-1: Insulin Induced Gene-1

IRGM: Immunity-related GTPase Family M Protein

LDL-C: Low Density Lipoprotein-Cholesterol

LOBs: Linoorbitides

LPS: lipopolysaccharide

LY: Lucifer Yellow

MAPK: Mitogen-Activated Protein Kinase

MDA: Malondialdehyde

MTX: Methotrexate

NCF-2: Neutrophil Cytosolic Factor 2

NFκB: Nuclear Factor Kappa B

NNHPD: Natural and Non-prescription Health Products Directorate

NO: Nitric Oxide

NOD-2: Nucleotide binding Oligomerization Domain containing 2

NPC1L1: Niemann-Pick C-1 like 1 Protein

NPN: Natural Product Number

PEP: Phosphoenolpyruvate

Phe: Phenylalanine

PK: Pyruvate Kinase

PKM2: Pyruvate Kinase M2

PMNLS: Polymorphonuclear Cells

PPAR-α: Peroxisomal Proliferator Activated Receptor Alpha

PRR: Pattern Recognition Receptor

RIPA: Radio-Immunoprecipitation Assay

RNS: Reactive Nitrogen Species

RORγT: RAR-related Orphan Receptor gamma

ROS: Reactive Oxygen Species

SDG: Secoisolariciresinol Diglucoside

SECO: Secoisolariciresinol

sIgA: Secretory Immunoglobulin A

SOD: Super Oxide Dismutase

SREBP: Sterol Receptor Element Binding Protein

TEER: Trans-Epithelial Electrical Resistance

TFF: Trefoil Factor

TG: Triglyceride

TLR: Toll-Like Receptor

TNF-α: Tumor Necrosis Factor-alpha

TPD: Therapeutic Products Directorate

UC: Ulcerative Colitis

WHO: World Health Organization

ZO: Zona Occludin

CHAPTER 1

Introduction and Literature review

1.1 INTRODUCTION

Flaxseed is one of the highly abundant agricultural products in Canada, especially in Saskatchewan[1]. Consumption of flaxseed is associated with an array of health benefits such as amelioration of disease markers in pathologies like cardiovascular-related disorders, diabetes, and cancer[2-7]. These health benefits have been attributed to an assortment of favorable reported biological effects including anti-inflammatory[8], antioxidant[9] and immunosuppressive activity[10], but the exact mechanisms of these activities are largely unknown. In dyslipidemia and diabetes, those activities were broadly studied for flax by the virtue of using standardized flax products as a nutritional intervention in human and animal models[2, 11, 12]. Conversely, immunosuppression in inflammatory diseases did not gain much attention in previous studies.

The health benefits of flaxseed consumption relate variably to a number of known bioactives including lignans, alpha-linolenic acid, and linoorbitides (LOBs)[13-16]. The limited known pharmacokinetic information regarding the flaxseed bioactives, lignans and cyclic peptides (LOBs), indicate a very limited oral bioavailability of these bioactives following oral consumption. The lack of extensive systemic absorption of the lignans and the large molecular weight of the LOBs (~1000 g/mol) suggest high concentrations and local effects on the gastrointestinal tract is possible. Very few studies have examined the role of lignans and LOBs on gastrointestinal function and how local effects might contribute to some of the health benefits associated with flax consumption[17, 18]. The overall objective of this research is to have compelling preclinical data to support the significance of flaxseed consumption to aid in pathologies like IBD and hypercholesterolemia. This will be carried by the study of local effects of flaxseed bioactives on intestinal function and to what extent this can be used to ameliorate chronic diseases like IBD and hypercholesterolemia. In addition, my research involves establishing the initial basis for their usage in a human clinical trial as an add-on supplement or nutritional intervention.

1.2 LITERATURE REVIEW

1.2.1 Flaxseed

Flax (*Linum usitatissimum*) is a name originating from a combination of a Celtic (lin; thread) and Greek word (usitatissimum; the most useful). Flaxseed has been used historically for several purposes like industrial oil, food, and as a source of fiber production[19]. In Canada, flaxseed is a major economic crop, especially in Saskatchewan, with an average production of 584 thousand tons yearly as the country`s leading province of flax production[20, 21].

Flaxseed has been widely studied for health benefits like improving lipid profiles, diabetic conditions, and in distinct types of cancer[22-24]. Flaxseed is composed of 28% dietary fiber, 41% oils, 21% proteins, lignans, and minerals along with some vitamins, carbohydrates, and negligible amounts of cyanogenic glycosides and other compounds[25]. Several components have been proposed to elicit the biological activities attributed to flaxseed, explicitly attributed to its three major components, omega-and alpha-linolenic acid (ALA), soluble and insoluble dietary fibers, and lignans. A fourth significant component of flaxseed, the cyclic peptides LOBs, have gained recent interest as potential bioactive compounds that add an important contribution to the health benefits of flax[10, 26]. These bioactive components exhibit a wide variety in chemical structure and their reported pharmacological activities encourage a more detailed and thorough scientific investigation of individual components for their significance in individual disease states.

1.2.1.1 Flaxseed historical applications

Flax is believed to be native to the areas eastern to Mediterranean basin and thought to be first domesticated in the Fertile Crescent[27]. Flax, which is also known as linseed when used in feed and clothing industry[28], has been widely used for several purposes including clothing (linens), dyes, paper and burial chambers dating back to about 3000 B.C.[13, 27]. Previously, flaxseed was recognized as a good source of industrial oil and fiber but has been replaced in the United States by cotton after the 1940's[27]. However, in the past two decades flaxseed gained major interest as a nutritional supplement or functional food due to the discovery of several pharmacologically active ingredients.

1.2.1.2 Flaxseed and health promoting components

Current knowledge of flaxseed extracts shows that the oil and seed coat have major components involved in health benefits[29]. Distribution of several flaxseed components is

summarized in table 1.1, where the main constituents are moisture, fiber, nitrogen containing compounds, and oil. Specific elements that are known to elicit health benefits like ALA is present in flaxseed oil products at significant levels, while the plant lignan, secoisolariciresinol diglucoside (SDG), which exists as a complex and produce mammalian lignans, enterodiol (ED) and enterolactone (ENL), through colonic fermentation, is present in the seed hull[22]. Flax contains considerable levels of soluble and insoluble fiber, which are considered beneficial for the bowel and for the maintenance of healthy intestinal microbiota[30, 31]. Finally, the previously discovered cyclic peptides[14, 15, 32], have undergone more significant investigation recently warranting a renaming of these peptides to LOBs[33]. Furthermore, LOBs extracted from flaxseed are presented in large variations; Burnett *et al.* quantified up to 25 different LOBs extracted from flaxseed[34]. These compounds are found mainly with the nitrogenous components in the cotyledons and embryo of the seed, and can be extracted with the lipophilic part of flaxseed[29].

Table 1.1 Different components of flaxseed and their distribution within the whole seed. Table adapted from Shim et al. (2014)[29].

Table 1. Flaxseed composition.

Constituent (%)	Whole seed	Cotyledons and embryo		Seed coat[a]	
		With fat	Without fat	With fat	Without fat
Moisture	7.13	4.31	NR[b]	7.89	NR[b]
Nitrogen	4.01	4.64	10.92	3.18	3.52
Oil	38.71	53.20	NR[b]	1.84	NR[b]
Fiber (soluble)	10.22	NR[b]	NR[b]	NR[b]	NR[b]
Fiber (insoluble)	30.41	NR[b]	NR[b]	NR[b]	NR[b]
Ash	NR[b]	3.38	7.95	2.99	3.31
Weight fraction	NR[b]	58.60	40.40	41.40	59.96
% of total oil			96.70		3.30

[a] Hull.
[b] Not reported.
Data taken from Hadley *et al.* (1992), Smith (1958) and source papers cited therein.

Inherently to natural products that are available in the market, there is a lack of standardization of constituents, and flaxseed is not an exception. In clinical and *in vitro* studies, we observe these variations as well. For example, Pan *et al* reports in a cross over trial using 360 mg/day lignan without identifying of the type of lignans included, neither their ratios in the final product, creating a difficulty to reproduce their results in other labs[2]. This comes in addition to the natural variations

in different cultivar in different areas at different times of cultivation[35]. With this variation in the bioactive components of flaxseed, it is a pivotal process to refine flax products and standardize natural products in order to attain maximum benefit and proper correlation between observed effects and content utilized. Typical drug regimens are composed of one molecule. Conversely, natural products are more frequently produced as a mixture. Mixtures in natural products are believed to elicit multiple pharmacological activities and target several targets, providing superior, safe health promoting activities. The term used for multiple targeting, in this case, is "multi-targeted phytotherapeutics"[36]. Owing to the complexity of diseases like cancer, diabetes, hypertension and autoimmune disease, it is believed that targeting several pathways is a better substitute to the 'one drug-one target' classic approach[37]. At the core of phytomedicine is the understanding that multicomponent therapy imparts strong synergistic or additive effects, where historically, extraction processes of the natural medicine enrich multiple components rather than a single component[38].

1.2.2 Pharmacological mechanisms of flaxseed derivatives

Current knowledge of flaxseed extracts reveals a strong association in several chronic diseases. These activities can be attributed to significant anti-inflammatory activity, immunosuppressing potential, antioxidant activity and a debatable sex hormone modulating effects.

1.2.2.1 Anti-inflammatory potential

The anti-inflammatory effect of flaxseed is reported in the context of post-menopausal women, lipid profile abnormalities, and cardiovascular diseases[8, 39-41]. The exact mechanism is yet to be evaluated for each component of flaxseed, but reports on cyclooxygenase-2 (COX-2) inhibition and its inflammatory cascade is abundant in literature. Flaxseed ALA is specifically associated with COX-2 inhibition[42], in addition to inhibition of nitric oxide production and via Nuclear Factor Kappa-B (NF-κB) and Mitogen-Activated Protein Kinase (MAPK) Pathways[43]. With respect to lignans, the mammalian lignan metabolite, ENL, is similarly associated with NF-κB modulation and subsequent inhibition of inflammatory cytokines[44].

1.2.2.2 Immunosuppression

Flaxseed cyclic peptides (LOBs) are a major contributor to the immunosuppressive activity of flax[45, 46]. LOBs are not as well studied as compared with other flaxseed constituents. One LOB,

LOB-A was synthesized and evaluated among other LOBs for immunosuppressant activity in mouse splenocytes and suppressed its proliferation[46]. This superior activity for LOB-A was attributed to the amino acid phenylalanine (Phe) at position number 4 in the cylic-peptide[46]. In addition, LOB-A has undergone limited investigation *in vitro* and in an animal model of humoral immunity to assess its immunosuppressive effects. In this model, LOB-A combined with methotrexate (MTX), a well-known immunosuppressant used in post xenograft transplant surgeries, improved graft rejection outcomes[47]. This immunosuppressive activity was compared to cyclosporine A (CsA) capacity to inactivate T-lymphocytes, which are major players in graft rejection, suggesting that co-administration of LOB-A and CsA will reduce the dosing requirements of CsA[10] and thus protect from potential side effects. The exact mechanism of action for LOB-A is not entirely understood. It is postulated to bind to cyclophilin A, a protein that is believed to inhibit calcineurin production that, in turn, prevents graft rejection[10]. Siemion *et al* studied LOB-A and compared it against several other structural analogues like antamanide, cycloamanides, hymenistatin, hymenamides and Cyclosporin, to understand what segment of the peptide results in the immunosuppressive activity[48]. Results from this study confirmed the importance of Phe, but also emphasized the need for a Proline-Hydrophobic amino acid-Phe sequence as an important player in this activity[48]. Finally, as an application for the use of LOB-A as immunosuppressant it has been investigated for its antimalarial activity and found to have advantageous activity[49].

1.2.2.3 Anti-oxidant activity

The flaxseed lignans demonstrate anti-oxidant activity which is attributed to their diphenolic nature[50]. The antioxidant activity of lignans is due to their capacity to scavenge free radicals through donation of a hydrogen (primary antioxidant function)[51] or by modulation of superoxide decomposition and enzyme regulation (secondary antioxidant mechanism)[52]. For lignans, anti-oxidant activity is reported to be highest with the mammalian lignans, ENL and END, and lower with SDG[53]. The lignans of flaxseed have demonstrated antioxidant activity in *in vitro* models such as the 2,2`-azobis (2-amidinopropane) dihydrochloride (AAPH) peroxyl radical-induced DNA damage model[52] and polymorphonuclear leukocytes (PMNLs)[54].

The primary antioxidant activities of the plant lignan, SDG, and the mammalian lignans, END and ENL, were evaluated in both lipid and aqueous *in vitro* systems. The three lignans significantly (*p-value* ≤ 0.05) inhibited linoleic acid peroxidation at both 10 and 100 μM over a 24-48 h of

incubation period[53]. This antioxidant behavior is likely chemically attributed to benzylic hydrogen abstraction and potential resonance stabilization of phenoxyl radicals in an aqueous environment and occurs at concentrations that are considered to be achievable *in vivo*[52]. Whether it is benzylic hydrogen abstraction or resonance stabilization, most studies showed the need for micromolar concentrations of lignans to elicit an antioxidant effect[52-55].

1.2.2.4 Sex hormone modulation

Lignan effects on sex hormones stems from its structural similarity to estrogen[56]. Further analysis on lignan binding to sex steroid binding protein ranked the lignans with highest to lowest inhibitory effect as follows: ENL followed by Nordihydrogaiaretic acid, Equol and Genistein[56]. This effect granted the nomenclature of phytoestrogen to lignans.

Sex hormone activity modulation is unique to the lignan component of flaxseed. In a randomized cross-over study performed in post-menopausal women, flaxseed fortified diet reduced the concentrations of circulating 17-β estradiol, estrone and increased prolactin[57]. Also, significant positive correlation (participant on conventional diet) between ENL excreted in urine and Sex Hormone Binding Globulin (SHBG) in blood was observed[58]. Binding of lignans to SHBG is believed to disrupt its interaction with dihydrotestosterone, which is thought to reduce proliferation in prostate cancer cells[59, 60]. In addition, inhibition of aromatase (an enzyme that converts testosterone to 17-β estradiol) was seen in human preadipocytes cells, suggesting a significant role in both estrogen and testosterone plasma concentrations[61]. Sex hormone modulation is associated with several cancers including breast and prostate cancers. Furthermore, they are involved in cholesterol metabolism and distribution in different body tissues.

1.2.3 Flaxseed in chronic diseases

Flaxseed intake has been linked to several benefits in chronic disease amelioration such as cancer, cardiovascular disease, and inflammatory conditions.

1.2.3.1 Colorectal, breast and prostate cancers

Phytoestrogen containing natural products and standardized supplements such as flavonoids and lignans have shown significant improvement in cancer such as colon, breast and prostate[62, 63].

Colorectal cancer is considered the second most common type of cancer in men and third in women, and ranked as the third cause of cancer-related deaths worldwide[64]. Several modifiable risk factors can be controlled to reduce colorectal cancer risk, and among those factors are obesity,

physical activity and consumption of red and processed meat[65, 66]. As a cancer related to the gastrointestinal system, it is pivotal to evaluate and understand the influence of dietary intake on such a neoplastic disorder. In colon cancer, unlike ω-6 PUFA, ω-3 fatty acids like ALA and the other mammalian lignans ENL and enterodiol (END) can reduce the size of the tumor in azoxymethane-induced colon cancer animal model[67]. ENL induced apoptosis and decreased cell proliferation in Colo-201 human colon cancer cells both *in vitro* and *in vivo*[68, 69] when administered in doses of 10 mg/kg three times weekly in athymic mice, assuring a considerable safety profile in such range. In contrast, clinical trials in humans did not support the correlation between END (no lignans were intentionally administered, just circulating dietary products) plasma concentration and risk reduction in colorectal cancer. Moreover, an increased risk was noted in the smoking population[70]. This discrepancy between clinical and bench results urges further investigation to understand the effect of flaxseed lignans against colorectal cancer.

In breast cancer, nutritional intervention with lignan-containing derivatives has been demonstrated clinically to reduce circulating tumor markers, size and metastasis, especially in postmenopausal populations in contrast to premenopausal[5]. Generally, clinical trials assess circulating levels of the mammalian lignan ENL as a metabolite for the parent lignan, SDG[71, 72]. One of those prospective clinical studies (evaluating lignans from conventioanl diet) pursued a follow-up of ENL concentrations for a median of 6.1 years to find that postmenopausal women with ENL concentrations equal to or exceeding 21.4 nmol/L lived longer than those who had circulating ENL concentrations less than 17 nmol/L. In general, the clinical literature identifies a possible important role of dietary intervention in chronic diseases.

The prostate is a gland related to the endocrine system and due to its involvement in sex hormone production and secretion, the prostate is affected by estrogen receptor modulation. As weak phytoestrogens, lignan consumption might modulate hormone receptor function in prostate cancer[73], but thus far clear, conclusive data does not exist. Prostate cancer and flaxseed studies have not been consistent globally. Clinical trials in the United States positively correlate ENL (from diet) levels with reduced prostate cancer risk[74]. In Nordic countries, though, this correlation was insignificant, but with several drawbacks on study design that included the lack of dietary monitoring and follow up[75]. Plasma insulin-like growth factor-I (IGF-I) has been associated with increased risk of prostate cancer[76]. In a rat model of breast cancer, flaxseed extract showed unique

ability to reduce IGF-I suggesting that modulation of IGF-I might be another possible mechanism in addition to estrogen receptor modulation to reduce the risk of prostate cancer[76].

Several mechanisms of action were proposed to explain the activity of flaxseed extract in cancer, including inhibition of growth factors, alteration of angiogenesis, and increased apoptosis through modulation of estrogen metabolism and estrogen receptor-related growth pathways[77]. Flaxseed oil that is rich in ALA is thought to provide its effect through activation of transcriptional factors like NF-κB and peroxisomal proliferator-activated receptors (PPARs) that would, in turn, influence cellular proliferation, angiogenesis, and differentiation. Moreover, increased peroxidation of ALA and other related ω-3 Poly Unsaturated Fatty Acids (PUFA), Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA), is assumed to be responsible for reduced tumor size and growth[77]. This same study also revealed a vital outcome, where co-administration of either flaxseed or its oil and anti-cancerous agents will not negate the activity of drugs like Tamoxifen and Trastuzumab, but rather enhance their effectiveness during the course of treatment.

Despite that mechanistic variations of biological activities in flaxseed, different bioactives of flax have been reported to have distinct effects in different tumor environments. For example, ALA is more efficient in MCF-7 breast cancer when estrogen levels are high, while SDG is more potent when estrogen levels are low[78]. These pronounced variations in mechanisms and tumor susceptibilities suggest the possible capacity of flaxseed bioactives to be involved in multiple pathways of protection against cancer, in particular, and possibly other chronic diseases.

In addition to studies involving breast, prostate, and colorectal cancer in animal models and human clinical studies, flaxseed lignan consumption is positively correlated with reductions in other tumor models. For example, in a dimethylbenzanthracene tumor induction animal model, administration of the flaxseed lignan, SDG, resulted in a 37% reduction in the number of tumors per tumor-bearing rats[4]. Collectively, these consistent reports in animal tumor models favor the translational potential of flaxseed utilization in the animal model as a means to extrapolate its benefits to humans.

1.2.3.2 Flaxseed and cardiovascular health

1.2.3.2.1 Flaxseed and risk factors of cardiovascular disease

Cardiovascular disease (CVD) is a leading cause of mortalities worldwide. It has two major classes, Coronary Heart Disease (CHD) and Coronary Artery Disease (CAD), both associated with

several chronic diseases like diabetes, atherosclerosis, hypertension and dyslipidemia with its variations[79]. Risk factors responsible for CVD include smoking, diet, gender, blood pressure, and chronic inflammation[80]. The use of flaxseed products to modulate risk factors of cardiovascular disease has undergone extensive study in *vivo* and *in vitro*. Among those risk factors are blood pressure, glucose levels and cholesterol[11, 12, 30, 81-85].

1.2.3.2.2 Cardiovascular health and hypercholesterolemia

Hypercholesterolemia has been widely studied from a nutritional aspect as a significant cardiovascular disease risk factor, and the long-term correlation between nutrition, obesity and CVDs is well established[86]. Nutritional intervention with low-carbohydrate diet improved atherogenic dyslipidemia even when weight loss is absent[87]. Similarly, National Cholesterol Education Program in Canada approved lifestyle change that embodies exercise and decreased intake of saturated fatty acid in addition to supplements like fish oil, oat bran and plant sitosterols as these interventions are considered to be efficacious in reducing cholesterol levels in those diagnosed with dyslipidemia[88].

Several therapeutic classes are in the market to help reduce circulating harmful cholesterols and lipids including statins, fibrates, bile acid binding resins and cholesterol absorption inhibitors. Statins are first-line agents in the treatment of dyslipidemias. Statins act by inhibiting HMG-CoA reductase, the enzyme that controls the production of cholesterol[89]. Only 50% of patients attain their target cholesterol levels upon the administration of statins, with differential responses among different statins[90]. This drawback combined with the side effects associated with statins like rhabdomyolysis, myositis, neuropathies and liver function alteration compel further investigation into safer and more effective medications in an attempt to reduce mortalities and morbidities associated with dyslipidemia[89].

Fibrates are peroxisomal proliferator-activated receptor alpha (PPAR-α) agonists. PPARs are nuclear transcription factors that induce the expression of several proteins and genes that are important in lipid homeostasis[91]. Fibrates are used to treat hypertriglyceridemia and mixed hyperlipidemia as they can reduce TGs and increase HDL-C[92]. Fibrates provided the maximum benefit when they are added to statins in order to improve HDL-C[93].

Bile acid sequestrants act by binding to bile acid in the gut resulting in a reduction of low-density lipoprotein-cholesterol (LDL-C) by increasing the production of LDL-C receptors in the liver. Receptor upregulation increases liver capacity to clear cholesterol metabolites. However, the

bile acid-binding resins interact with the absorption of lipid soluble vitamins and other critical nutritional elements like iron, folic acid, and magnesium. This interaction may require more attention in patients suffering from imbalances in those vitamins and minerals[92].

Ezetimibe is a cholesterol absorption inhibitor that acts by binding to Niemman-Pick C-1 like 1 (NPC1L1) protein. It is used as an add-on medication to statins in order to improve LDL-C levels[94]. Ezetimibe is very well tolerated and is not associated with side effects that would affect its adherence or long term functionality, but yet not thoroughly studied [92].

Lipid lowering agents have been available on the market for a while now, as they confer favorable effects to reduce the incidence of atherosclerosis and consequently CVD events in half of the population suffering from impaired lipid profiles (case specific to statins)[95]. The urge to bring more lipid-lowering agents is justified by the need to provide more effective drugs with reduced potential of adverse events[92].

1.2.3.2.3 Interventional cholesterol reduction studies

A- Animal studies

Different types of studies have been performed in animals, where either flaxseed extract complex or a purified lignan component was administered. Prasad *et al.* reported that chronic administration (4 months) of flaxseed complex to rabbits resulted in reduced serum lipids and aortic atherosclerotic events[84]. Similarly, Syrian Hamsters treated for 120 days with flaxseed extract (contains fiber, ALA, and lignans) showed reduction of plasma cholesterol but surprisingly increased triglyceride (TG) levels[83]. Previously published research in our laboratory investigated the effect of the lignans, SDG and secoisolariciresinol (SECO), in hyperlipidemic rats, after induction of nutritional hypertriglyceridemia by 10% fructose and hypercholesterolemia by 1% cholesterol. This study concluded that flaxseed lignans could reduce LDL-C and total cholesterol in serum, but had no effect on TG levels, which is consistent with sesamin (a sesame seed lignan) results in an LDL-receptor knock out animal model[12]. The exact mechanism of action is not understood, but hepatic regulation of lipid homeostasis through well-known possible targets were excluded[12].

Enterolactone was investigated in metabolic syndrome. Urinary excretion of lignans, and specifically ENL, was positively correlated with increases in HDL-C and reduction in LDL-C but had no influence on other parameters like waist circumference or blood pressure[96]. Previous studies have directly correlated the concentration of enterolignans (ENL and END) excreted in

urine to increased HDL-C levels and reductions in TG, while LDL-C levels were not substantially affected in 1492 U.S adult hypercholesterolemic patients[97]. Furthermore, a dose-response correlation was observed between SDG extract consumption and blood levels of SECO, ENL, and END and cholesterol lowering capacity[11]. SDG might protect liver function as oral SDG administration is correlated with decreased Gamma Glutamyl Transpeptidase (GGT) levels (an enzyme that is activated in liver injury)[82]. Furthermore, in clinical trials of moderately hypercholesterolemic patients (see table 1.2), dosing with SDG improved lipid profiles through significant reductions in LDL/HDL ratios, suggesting that it has multiple effects on cholesterol transport in the systemic circulation.

B- Human interventions

Table 1.2 Summary of clinical experiments that used flaxseed lignan enriched products to observe changes in lipid profile.

Reference	Dose	Study population	Duration in weeks	N	HDL-C change	LDL-C change	Tri-glycerides	Total cholesterol
Pan *et al.* (2007)[2]	SECO 124 mg (360 mg SDG) / capsules	Diagnosed with type 2 Diabetes and >2.9 mmol LDL-C	12	73	-0.77 mg/dL	-4.25 mg/dL	-17.70 mg/dL	-6.56 mg/dL
Hallund *et al.* (2006)[98]	SECO 172 mg (500 mg SDG) / muffins	Healthy post-menopausal women	6	22	-1.54 mg/dL	-7.72 mg/dL	3.5 mg/dL	-8.88 mg/dL
Zhang *et al.* (2008)[11]	SECO 103 mg (300 mg SDG) / tablets	Hypercholesterolemic Patients	8	18	-3.86 mg/dL	-28.6 mg/dL	-46.0 mg/dL	-39.8 mg/dL
Cornish *et al.* (2009)[99]	SECO 187 mg (543 mg SDG) / tablets	Men over 50 and post-menopausal women	26 26	92 49	N/A	N/A	N/A Increased	No effect

1.2.3.3 Inflammatory diseases (Inflammatory Bowel Disease (IBD)

1.2.3.3.1 Nutritional Intervention in IBD

Among the numerous biological effects of flaxseed bioactives, these compounds have been reported to exert anti-inflammatory effects. This activity is considered important in diseases that have inflammation as a critical player in their pathologies like cancer, cardiovascular disease, and inflammatory bowel disease[52].

IBD is viewed by both clinicians and patients to be influenced directly by the type of dietary regimen patients are adhering to. Yet, there are no comprehensive recommendations on what type of diet might reduce antigen exposures or alter gut microbiota to improve intestinal barrier integrity[100]. Different types of food have resulted in a variable effect on intestinal inflammation. Refined sugar and high fat/high protein diets are associated with exacerbated intestinal inflammation[101]. Dietary linoleic acid, which is one of the main components found in flaxseed, has been related to the improvement of ulcerative colitis in human clinical trials[102]. On the other hand, dietary intake has an indirect effect on intestinal microbiota, which in turn significantly influences barrier integrity and immunity[31, 103]. To this date, there are no clear nutritional intervention guidelines that help mitigate signs and symptoms of IBD.

1.2.3.3.2 Inflammatory Bowel Disease (IBD).

Inflammatory bowel disease (IBD) is characterized by idiopathic chronic inflammation along the intestine with varying locations and clinical presentations and is subdivided to Ulcerative Colitis (UC) and Crohn`s Disease (CD). IBD's unclear multifactorial etiology could be correlated to variable environmental, genetic, cellular, and immunological factors[104]. While UC manifestation is limited to the intestinal mucosa, CD inflammation extends transmurally. CD also shows histological granulomas and intestinal thickening localized to the proximity of the colon, unlike UC that has a more continuous presentation overall in the intestine[105].

Internationally, IBD has a prevalence rate of 369/100,000[106]. In Canada, 9,200 cases of IBD are diagnosed every year and 55% of cases are CD. In 2008, 201,000 patients (0.5% of population) suffered from IBD, 112,000 with CD and 88,500 with UC[107]. Canada ranks among the highest prevalence rates worldwide of IBD[108] and just after New Zealand with an incidence rate of 13.4/100,000 for CD[104]. Canada spends more than $1.8 billion yearly to cover costs associated with the health management of IBD patients. This cost is segmented to direct costs, which include

hospitalization, treatment, and clinic visits, and indirect costs that comprise more than one billion dollars to compensate lower labor participation, patient pocket money, and short-term work absence[107, 108].

Like other autoimmune diseases and chronic inflammatory disorders, IBD is characterized by complex interactions between anatomic, genetic factors, environmental conditions, and the immune system[109, 110]. Therefore, a multitude of defense mechanisms have evolved to protect the intestinal epithelium. Those mechanisms orchestrate to maintain a healthy intestinal epithelium with high integrity, and reduced inflammatory status is generally categorized under; 1- Innate and 2- Adaptive immune response[111]. Functional participation of innate immunity, adaptive immunity, and intestinal microbiota in the intestinal epithelium is critical for our understanding of both the pathogenesis and possible targets for bioactives that might alter the intestinal epithelial functions.

- **Innate Immune response**

The gastrointestinal capacity to secrete acidic juices, mucus, non-specific antibodies and digestive enzymes helps to kill non-host substances. In addition, peristaltic contractions reduce intestinal epithelial exposure time to noxious substances in the intestinal lumen[112]. Together these comprise the innate immune defense. Furthermore, production of chemokines and recruitment of white blood cells, followed by the release of cytokines and reactive oxygen species (ROS) will counteract the invading organism or particle, but such interactions may result in long-term activation of tissue damage and repair cycles, which will eventually trigger the adaptive immune response. To summarize, innate immunity in the intestine is primarily granted by intestinal mucosal layer, the intestinal epithelium barrier itself, oxidative stress, and immune cells like macrophages and dendritic cells[113]

A. Mucosal layer

Mucus is mainly composed of three principal components; mucin, a glycoprotein that is encoded by MUC2 gene and produced by goblet cells; trefoil factors (TFF), a group of polypeptides that are protease resistant; and secretory IgA (sIgA). Disruption of those three factors is considered as a fingerprint for development of IBD[114].

The intestinal mucosa is considered the first line innate immunity checkpoint against invading microorganisms and noxious particles. Secretory antibodies (sIgA) and the viscosity of mucin lining the mucosa (due to mucin itself) that concomitantly assist in trapping microbes and exposing them to antimicrobial agents produced by the intestinal epithelial cells add to the first line defense

of the mucosa[114, 115]. This function is boosted by pattern recognition receptors (PRR), such as toll-like receptors (TLR), retinoic inducible gene I like receptor (RLR), and nucleotide oligomerization domain-like receptors (NLR), that will modulate the production of mucus and simultaneously trigger the adaptive immune response[115, 116]. Each of those receptors will initiate different defense mechanisms. For example, the activation of NLR and TLR will induce NF-κB, which turns on the nuclear production of cytokines and pro-inflammatory responses, a function that will elicit an adaptive immune response[116]. As well, pattern recognition receptors functionally initiate intestinal epithelial secretion of antimicrobial agents like defensin, calprotectin, cathelicidins, and antimicrobial polypeptides, an important component of the innate immune system[110, 117]. The mucus layer's inherent functionality is complemented with intestinal peristaltic contractions. Those contractions will help reduce the exposure time to noxious materials and, hence, mechanically reduce possible inflammatory triggers[111, 118].

B- Intestinal Barrier

The intestinal epithelium functions to protect the internal milieu of the body from luminal noxious materials like nutrients, microbes, and drugs. This protection is maintained through several mechanisms. Anatomically the intestinal epithelium is a polarized epithelium composed of a single layer of cells achieving a barrier between the external environment and the body and transport across this barrier is mediated through different mechanisms that allow selective movement of molecules based on their physicochemical characteristics[119]. The intestinal barrier maintenance is granted by tight junctions that prohibit paracellular flux of intestinally localized components and vice versa[120]. Tight junctions consist of clustered proteins including zonulin (ZO)-1, -2 and -3, cingulin and 7H6, and the transmembrane proteins occludin and claudin[121]. Furthermore, protection is maintained by the presence of a microenvironment in close vicinity to the epithelial cells composed of an unstirred layer of water, glycocalyx, and mucus layer containing secreted antimicrobial agents and immunoglobulins, which act as the first line of defense that is boosted by the innate immune response of the intestine[122].

C- Dendritic cells and Macrophages

Mononuclear phagocytes are divided into macrophages and dendritic cells. These cells physiologically emerge from the monocyte that comes from myeloid lineages. Macrophages are more prominent on both sides of the intestinal epithelium (basal and apical) and are maintained through the maturation of monocytes that are introduced to the intestine via the lymphatic system[123]. Dendritic cells (DCs) are surveillance cells able to recognize harmful and harmless

constituents in the gastrointestinal luminal space by sampling the exterior environment. Depending upon the stimulus, DCs either stimulate adaptive immune response or repress possible inflammatory status based on the antigen sampled[109, 124]. This possible activation of adaptive immunity is governed by interaction with tissues and cells like Pyere`s Patches (PP), Mesenteric Lymph Nodes (MLN), and Isolated Lymphoid Follicles (ILF)[111, 125]. Upon activation, differentiation of T-cells to Foxp3 + T-regulatory cells occur as a result of TGF-β and retinoic acid. This, in turn, leads to the production of IgA, which is a critical part of the innate immune response[126-128].

DCs vary significantly depending upon the type of integrin attached to the DC. CD103+ DC are thought to be the cells responsible for sampling antigens, and subsequent up-regulation of inflammatory markers and maturation of lymphoid structures rich with T-lymphocytes and B-lymphocytes[125, 129, 130]. Intestinal CD103+ DC are mostly involved in an immune suppressive activity in the intestine, as they can stimulate the production of thymic stromal lymphopoietin, which inhibits Th17 and allows the differentiation of Treg cells[109, 131]. Treg shares the responsibility of homeostasis; CD4+CD25+ Treg cells maintain homeostasis through screening of self and non-self-antigens, which was found to activate the anti-inflammatory cytokines IL-10 and TGF-β (see figure 1.1). Moreover, Treg cells regulate the expression of Foxp3+ transcription factor that influences the production of IL-10 that would aid in suppressing the immune respone[128, 132, 133].

Integrins are also expressed on macrophages and variants include CD11c, 11b, and CX3CR1 macrophages[134, 135]. The subtype (CX3R1) is characterized by a relatively short half-life, which is estimated to be three weeks[136]. This subtype is responsible for sampling from the intestinal lumen while they reside in lamina propria. This process is called trans-epithelial sampling, where dendritic extensions from mature macrophages paracellulary pass toward the lumen and sense non-host substances and samples them, a function that is critical in maintaining the balance between intestinal microbiota and host defense[124]. Depending on the antigen source whether it is bacterial, parasitic or others, different cellular lineages will be activated accordingly. The following figure summarizes the interaction between APCs, cells activated and cytokines produced and their corresponding effector cells that will be activated (Figure 1.1).

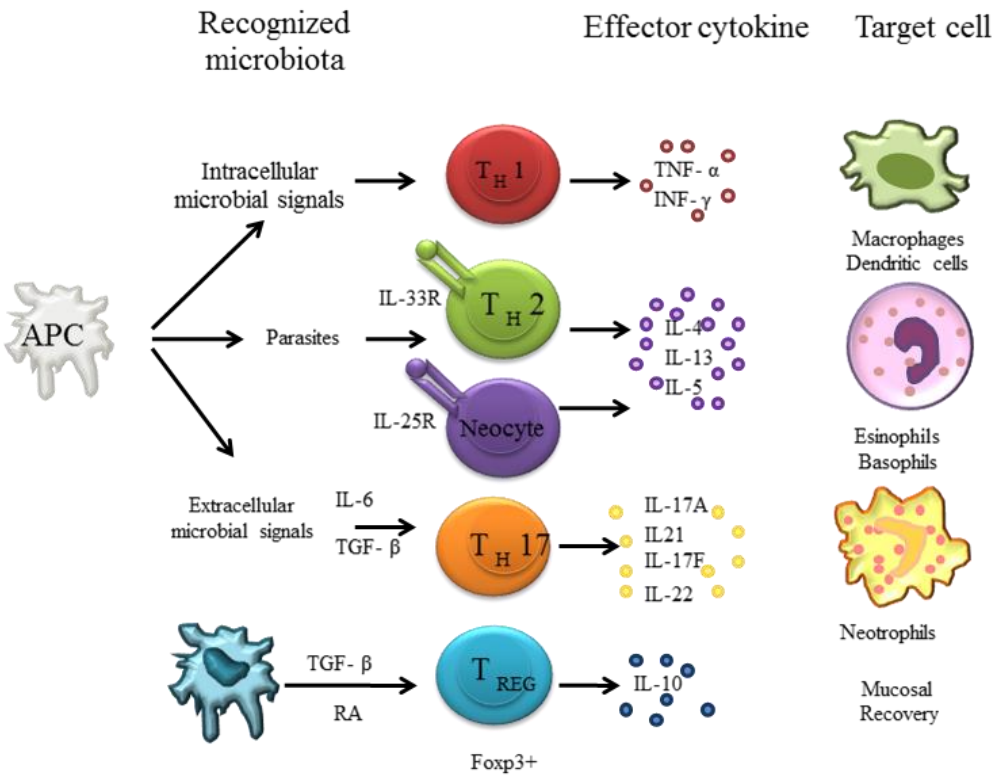

Figure 1.1 Depiction of different cells that are involved in maintaining immunological homeostasis in the intestine. Several noxious materials will initiate different immunological responses. Intestinal Epithelial Cells (IEC) join with Antigen Presenting Cells (APCs) to stimulate both innate and adaptive immune responses and result in the production of cytokines and inflammatory mediators. The predominant effect of mucosal recovery is highly attributed to the activity of Treg cells and their corresponding cytokines, IL-10 and TGF-β, in addition to Foxp3+ transcriptional pathway.

D- Oxidative stress and IBD

Prolonged production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) signaling might magnify the oxidative process and, thus, reduce barrier integrity and eventually lead to the pathogenesis of IBD[137]. Oxidative and nitrative damage to cells occurs through DNA breakage, damage, and mutation emergence. DNA damage will progress toward carcinogenesis in animals and humans; this damage is caused by continuous chronic exposure to radicals where natural cellular repair mechanisms start to malfunction[138]. Several enzymes and endogenous molecules with redox activity (e.g. superoxide dismutase-1 (SOD-1), catalase, glutathione peroxidase-1 and 2, reduced glutathione, ascorbic acid, carotenoids) are clinically correlated to IBD as biomarkers for disease severity[55, 139-142]. Also, thioredoxin and aquaporin have reportedly increased activity in response to inflammation and are suggested to be a protective mechanism

against persistent inflammatory status in IBD[142, 143]. Intestinal epithelial cells, macrophages, and lymphocytes are all involved in the generation of ROS and RNS as a part of their natural defense mechanism[137].

Intestinal epithelial cells are equipped with Toll-like receptors (TLRs) that are capable of identifying pathogenic organisms, and signaling activation of cytokines and chemokines through the activation of NF-κB[144, 145]. In clinical scenarios of ischemia and reperfusion, a state of oxidative stress is developed and results in tissue damage[146]. Interestingly, the TLR-4 agonist, Lipo-polysaccharide (LPS), will provide protection in intestinal ischemia by reducing intestinal permeability, lipid peroxidation, and glutathione depletion[147]. The dual role of LPS and TLRs suggests a homeostatic role of TLRs to maintain oxidative stress pathways.

Macrophages produce high amounts of peroxynitrite ($ONOO^-$) and H_2O_2 in response to an inflammatory stimulus[137], in addition to other cytokines like TNF-α and IL-1β[148, 149]. This excitatory state is mainly inhibited by IL-10, which results in reduced heme oxygenase and TNF-α expression through p38 MAPK pathway[150]. This generation of different oxidation generators by macrophages is attributed to the pathogenesis of IBD[151, 152]. Therefore, macrophages are crucial in initiating oxidative stress, inflammation, and later in recruiting other inflammatory mediators.

Lymphocytes that are resident in the lamina propria (the layer between intestinal epithelium cells and muscularis mucosa) are recognized to play a role in thioredoxin expression[153]. Thioredoxins are the major cellular protein disulfide reductases. They serve as electron donors for enzymes such as ribonucleotide reductases, thioredoxin peroxidases (peroxiredoxins), and methionine sulfoxide reductases, and, therefore, thioredoxins have multiple biological functions like protection against oxidative stress, control of growth, and activation of cytokine production[154]. Upon stimulation of the lamina propria, T-cell activation of the enzymatic activity of thioredoxin is elevated, which modulates oxidative stress in the intestine[153]. Increased expression of thioredoxin results in increased resistance to the immunosuppressive activity of CsA, and, therefore, again confirming the sensitivity of the intestinal microenvironment[153].

Although basal levels of nitric oxide (NO) are necessary for the maintenance of intestinal homeostasis, enhanced NO production is also correlated with IBD progression. Two enzymes are involved in the production of NO, inducible nitric oxide synthase (iNOS) and eNOS, which is produced constitutively in many cell lineages[137, 155, 156]. When challenged by dextran sodium sulfate (DSS: colitits model), mice that are deficient in both iNOS and eNOS showed a progression

of the disease and higher disease activity scores[155, 157]. In humans, iNOS deficiency is also considered a marker for chronic progression of disease activity, increasing leukocyte adhesion and disruption of barrier integrity[158].

- **Adaptive Immune response**

Both innate and adaptive immune responses often function at the same time and behave simultaneously to achieve homeostasis in the intestine and prevent the emergence of unnecessary excessive inflammation. A significant crosstalk between different cell types including epithelial, endothelial, and leukocytes is necessary to assure immunological homeostasis in the gastrointestinal tract.

Historically, T-cells were identified to be a major player in autoimmune diseases, and Th1 and Th2 were explicitly described to be involved in CD and UC, respectively[118]. Initially, IBD was thought to be solely driven by Th1 activation. This cell type induces the production of IL-12p40, a cytokine that has been associated with chronic inflammatory diseases in general[118, 159]. Recently, the activation of a different type of differentiated T helper cells, the Th17, is believed to be also critical in autoimmune diseases[160, 161]. Th17 activation in addition to Th1 activation leads to the production of the structurally similar cytokines, IL-23 and IL-12 (IL-23 is a heterodimeric cytokine that belongs to IL-12 family that is composed of Il-23p19 combined IL-12p40 subunits)[118]. This activation is mediated by IL-6 and TGF-β, which upon activation induce the expression of retinoic acid-related orphan receptor γt (RORγt), which, in turn, increases the expression of IL-23[159].

Interleukin-23 (IL-23) and its producer cells, Th17, were correlated to several types of autoimmune diseases including arthritis, brain, and intestinal colitis[160, 162-164]. Currently, the activation of Th17 is considered a major player in adaptive pathogenesis of IBD. Therefore, several therapeutics are currently in clinical trials to target IL-23[164, 165].

Another important player in both adaptive and innate immunity is B_{reg} cells, especially those marked with the integrin CD1d. These cells are abundant in the gastrointestinal tract and lymphoid tissue and suppress the immune response through secretion of the anti-inflammatory cytokine IL-10[166]. IL-10 achieves its anti-inflammatory protective effect by upregulating the STAT3 pathway and IL-1 expression[150].

- **Microbiota and intestinal immunology**

A growing trend of experiments and hypotheses exist suggesting that intestinal commensal microorganisms play a role in initiating, maintaining, and determining the phenotype of IBD[167].

The precise function of the microbiota in the etiology of the disease is largely unknown; however, four mechanisms have been postulated to explain the complex relationship between commensal microorganisms and IBD: 1- Dysbiosis (microbial imbalance) of conventional microbiota, 2- Induction of intestinal inflammation by pathogens and altered microbiota, 3- Host genetic defects of maintaining homeostasis, and 4- Malfunctioning host immunregulation[167]. All these mechanisms will result in a persistent antigenic activation for T-lymphocytes and macrophages[168].

Pathologically, IBD is more predominant in the colon and distal ileum, which are both highly populated with commensal microbiota[169]. Dysbiosis in IBD favors an 8-fold increase in the mucosal-associated bacteria (aerobic and anaerobic) in patients with IBD in comparison to asymptomatic patients[170]. In addition, the diversity of microbiota in active IBD patients is considerably reduced; this presents as a contraction of *Firmicutes* and *Bacteroidetes* and an expansion of *Proteobacteria*[169].

Induction of inflammation is considered as a consequence of dysbiosis, but more profoundly it is attributed to pathogens[171]. Intestinal inflammation is induced by species like *Clostridium Difficile* and it's associated toxin-A that is capable of reactivating IBD through inducing apoptosis in intestinal epithelial cells[172]. *C. difficile* infection, in particular, is clinically typical upon usage of broad-spectrum antibiotics, which results in reduced microbiota populations and hence considered to be a potential trigger for excessive inflammation.

Host genetic polymorphism and mutation are able to influence microbiota populations thus causing dysbiosis. The most frequently reported genetic polymorphism associated with IBD is related to the intracellular pattern recognition receptor, NOD-2 (nucleotide oligomerization domain-2)[173]. Once mutated, a reduced intestinal capacity to produce cytokines is observed and thus an increased population of commensal bacteria, and subsequent dybiosis[171]. In addition to NOD-2, other disease-related genes like ATG16L1, NCF-2 and IRGM would be anticipated to reduce luminal and intracellular bacterial killing, and therefore result in dysbiosis that is critical in IBD[174, 175].

Intestinal microbiota also has a beneficial effect on intestinal epithelium integrity. Clusters IV and XIVa of *clostridium* bacteria induce the activation of Foxp3+ Treg cells in the colon, which helps the production of IL-10 and TGF-β; both are previously discussed as anti-inflammatory cytokines[176]. *Bacteroides fragilis* is involved in symbiosis and expressing IL-10 and TGF-β through the interaction between LPS of *Bacteroides* and the (Pathogen recognition receptors)

PRRs the TLR-2[177]. Interestingly, *Bifidobacterium longum* is capable of secreting acetate as a nutrient for intestinal epithelial cells, a protector against *E-coli* infections and an enhancer of barrier integrity[178, 179].

1.2.3.3.3 Current IBD treatments and their limitation

IBD is attributed to notably increased levels of pro-inflammatory immune modulators like TNF-α and INF-γ and specific interleukins. These molecules became targets for different pharmacologically active candidates to treat IBD. Currently, IBD intervention involves the use of salicylate derivatives, oral corticosteroids, immunosuppressive agents, anti-TNF-α biologics, antibiotic therapy, and surgery in refractory cases[180].

A multicenter study to investigate IBD disease outcomes in patients across Europe showed that usage of conventional therapies (mentioned above) will result in complete relief of complaints reported in 48% of UC patients, and only in 35% of patients with CD[181]. In adherence predictor studies, it was found that patients suffering from IBD and on immunosuppressant medications are more likely not to adhere to their medications[182]. In addition, self-reported non-adherence for all IBD medication reaches up to 21% in certain populations[183], which generally ranges between 30-45%[184]. Patients' belief and fear of side effect were reportedly listed to be one of the leading causes of non-adherence[183-185].

In regards to cost, Canada spends more than $1.8 billion yearly to cover costs associated with the direct and indirect health management of IBD[107]. In the United Kingdom, individual patient costs ranged from £73 to £33 254 per six months treatment period, with a mean of £1256 for colitis and £1652 for Crohn's disease[186]. The addition of Infliximab biologic to patients` management in the United States costs a commercial insurer an average of $2,800 for a single dose (every 4-6 weeks)[187]. Both public and private sectors suffer from the current high cost of IBD management in general and biologics specifically.

In addition to therapeutic challenges, there is a considerably important challenge in staging and diagnosis of disease course. Currently, several biomarkers and their combinations are considered to aid diagnosis of IBD including C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), fecal lactoferrin, fecal calprotectin and calgranulin C[188]. In addition, biomarkers to distinguish between IBD phenotypes have also been utilized in clinical practice to help assess the severity of disease including anti-*Saccharomyces cerevisiae* antibody, anti-glycoprotein-2, anti-granulocyte macrophage colony-stimulating factor, anti-neutrophil cytoplasmic antibody, anti-

mannobioside carbohydrate IgG antibody, and anti-CBir1[189]. However, these biomarkers lack sufficient sensitivity, and specificity[190-192,193, 194] and endoscopy remains the gold standard to diagnose and to stage disease severity in IBD patients [189, 195].

1.2.3.3.4 Use of Flaxseed to mitigate intestinal inflammation

Several lignans such as Manassantin A and B and saucerneol D and E reduced TNF-α expression in LPS induced inflammation (In BV-2 microglial cells) in a dose-dependent manner[196]. Following LPS stimulation of human peripheral blood lymphocytes, ENL and END demonstrated a concentration dependent inhibitory effect on Nf-κB with ENL showing greater potency than END and thus reducing the potentiation of inflammation[44]. Furthermore, flaxseed derived products that contained ALA were tested against two different expiremental models of colitis to show positive outcomes on disease activity indices[197]. Although several studies have shown the possible IBD amelioration potential by lignans, the literature is not as rich when it comes to flaxseed lignans or LOBs. Studies that used flaxseed extract showed conflicting results, which could be attributed to the extract type used in those studies. For example, in an acetic acid model of colitis flaxseed oil was not equivalent to fish oil in regards to amelioration of mucosal inflammation[17]. Similarly, Zarepoor *et al.* reported exacerbation of acute mucosal injury after three weeks of different flax extracts (10% flaxseed, 6% kernel and 4% hull). However, this conclusion was drawn for 10% flaxseed but not the other extracts that are considered richer sources of lignans, where improvement in disease activity scores was actually observed with the 4% hull extract source. These results encourage further investigation on multiple components of flaxseed in IBD to identify a flaxseed natural product with a primary effect of amelioration of IBD[18].

1.2.4 Flaxseed natural products (Chemoprevention Vs Chemotherapeutics)

With its reported pharmacological activities and role in chronic disease, flaxseed has gained public attention for its potential use as a sole therapeutic, adjuvant therapy, or chemopreventive agent. There is a public shift toward natural products as a safe source of medication due to ease of access and higher potential to target several pathways of activity with multicomponent plant extracts currently available on the market[198].

The use of health related product falls into two categories; chemopreventive and chemotherapeutic, where the latter typically represents the conventional process of drug discovery that has rigorous drug regulatory agency oversight on their utilization to help treat diagnosed disease. On the other hand, chemoprevention in cancer is defined as “the involvement of chronic

administration of a synthetic, natural or biological agent to reduce or delay the occurrence of malignancy[199]", a concept that can be extrapolated to other chronic pathologies. Regardless of its role as a chemopreventive or chemotherapeutic, a natural product that will be introduced to the market has to go through effectiveness and safety evaluations. These requirements compel more focused investigations with natural products to promote a better understanding of their biological activities, mechanisms, multicomponent effects, and safety in order to substantiate their health benefits with more robust scientific support.

1.2.5 Natural products regulations

Regulations for NHPs differ from one country to another. In Canada, the Food and Drugs Act 9 (FDA), governs the marketing of a NHP, which requires approval by the Food Directorate (FD), Therapeutic Products Directorate (TPD), or Natural and Non-Prescription Health Products Directorate (NNHPD), depending on what class of product is submitted for approval. Health Canada defines a NHP as a substance set out in Schedule I or combination of substances in which all their medicinal ingredients are listed in Schedule I under the umbrella of homeopathic medicine or traditional medicine that is manufactured, sold or presented in the use of: 1) restoring or correcting organic function in humans, 2) modifying organic function by either promoting or maintaining health, or 3) diagnosis, treatment or mitigation of disease, disorder or abnormal physical function in humans[200].

In order for a NHP to be licensed in Canada, it has to undergo rigorous refinements that are regulated in the Natural Health Product Regulation under FDA, which is divided to six parts; first part regulates the product license application, second part regulates the site licensing, third part regulates Good Manufacturing Practices (GMPs), fourth part involves regulations for clinical trials in human, fifth part includes general regulations and finally the sixth part which includes amendments and transitional provisions and coming into force regulations[201]. Once licensed for sale in Canada a NHP will be assigned either a DIN-HM (Drug information Number-Homeopathic Medicine) or eight digits Natural Product Number (NPN)[202]. In light of these regulations, and in order to supplement flaxseed extracts as chemopreventive products to help ameliorate chronic diseases it has to fulfill effectiveness, quality and safety parameters to gain such a status.

1.2.5.1 Flaxseed product quality

Standardized natural products extracts are defined by Kunle *et al* as a "high-quality extracts containing consistent levels of specified compounds, and they are subjected to rigorous quality

controls during all phases of the growing, harvesting, and manufacturing processes"[203]. Regulatory affairs bodies have not defined standardized natural products, therefore, a plethora of products in the market claims standardization without solid evidence.

For flax there are products that are standardized when it comes to content such as BeneFlax®. BeneFlax® is a previously marketed standardized flax product (~ 38% enriched with lignans) which went through the requisite quality and GMP processes. This product has gained Health Canada and US FDA approval as a standardized flaxseed source. Therefore, it is a potential candidate to be used chemopreventive entity that is able to provide symptom relief to moderate disease status or for the reduction in the risk for potential pathology.

1.2.5.2 Flaxseed effectiveness

The literature is replete with human and animal trial data that supports the possible effectiveness of flaxseed extracts. However, the positive effects for many of studies were not attributed to standardized products. To satisfy regulatory requirements, additional human and animal clinical trials involving standardized flaxseed products are necessary to support efficacy in various chronic disease conditions. Although alpha-linolenic acid has been the focus of many interventions involving flax, the mammalian lignans and LOBs are gaining more attention for further assessment due to more recent discoveries of their potential to mitigate inflammatory disease and modulate cholesterol homeostasis[10, 204].

1.2.5.3 Flaxseed safety

Generally, flaxseed intervention trials in humans did not report any adverse reactions or side effects[205, 206]. In addition, the flaxseed mammalian lignans (ENL and END) or their precursors (SECO) have not shown any genotoxic effects in the range of 20-100 µM concentration[207].

In a major safety randomized double-blind clinical trial where participants aged 49–87 years received flaxseed lignan (543 mg/day in BeneFlax®) equivalent to 200 mg of SDG, showed no signs of unintended side effects including hypoglycemia and hypotension during the six month period of the trial[206]. Similarly, when tested in benign prostate hyperplasic patients at two dosage regimens containing 100 mg and 200 mg SDG for eight weeks showed no adverse events at any point of the trial[205]. In addition to reported clinical trials, Health Canada approves that a minimum mass of 13 g of whole ground flaxseed is necessary to claim a cholesterol reduction capacity when ingested as food, which also fortifies the safety of the seed and its consumption at relatively high

levels[208]. Further understanding of flaxseed safety and efficacy requires a detailed understanding of the pharmacokinetic behavior of the bioactive components of flaxseed. However, despite these pronounced safety reports in human clinical trials, an animal study where flaxseed fed to pregnant rats resulted in the delivery of low birth weight pups with growth related developmental issues[209]. Such assessments in animals may provide modest evidence to restrict the use of flaxseed lignans in pregnant and breast feeding populations[209, 210].

1.2.6 Lignan and Linoorbitide pharmacokinetics

1.2.6.1 Lignan Pharmacokinetics

Our understanding of pharmacokinetic behavior is crucial in drug discovery; the more we know about drug pharmacokinetic behavior, the more reliable is our pharmacological utilization. Flaxseed lignans are not an exception, and their pharmacokinetic behavior is quite intriguing.

1.2.6.1.1 Absorption

In the plant, the lignan SDG exists as an oligomer with an average molecular weight of 4000 Da[211]. The oligomer units are reported to be linked directly to the glucosyl moiety of SDG by an ester linkage[212-214]. Once in the gastrointestinal tract, the ester linkages are putatively hydrolyzed to liberate SDG, with subsequent hydrolysis of the glycosidic bonds for the release of SECO. *In vitro* incubation of SDG with fecal material found different forms of aglycones, suggesting that the intestinal microflora can convert SDG into other aglycone forms[215]. Unabsorbed SECO continues to the colon where colonic microflora convert SECO to the mammalian lignans (ENL and END). SECO, ENL, and END become available for absorption, which is supported by their presence in the systemic circulation[216, 217]. The structural differences between mammalian lignans and plant lignans are mainly the methyl groups on phenolic rings (Figure 1.2).

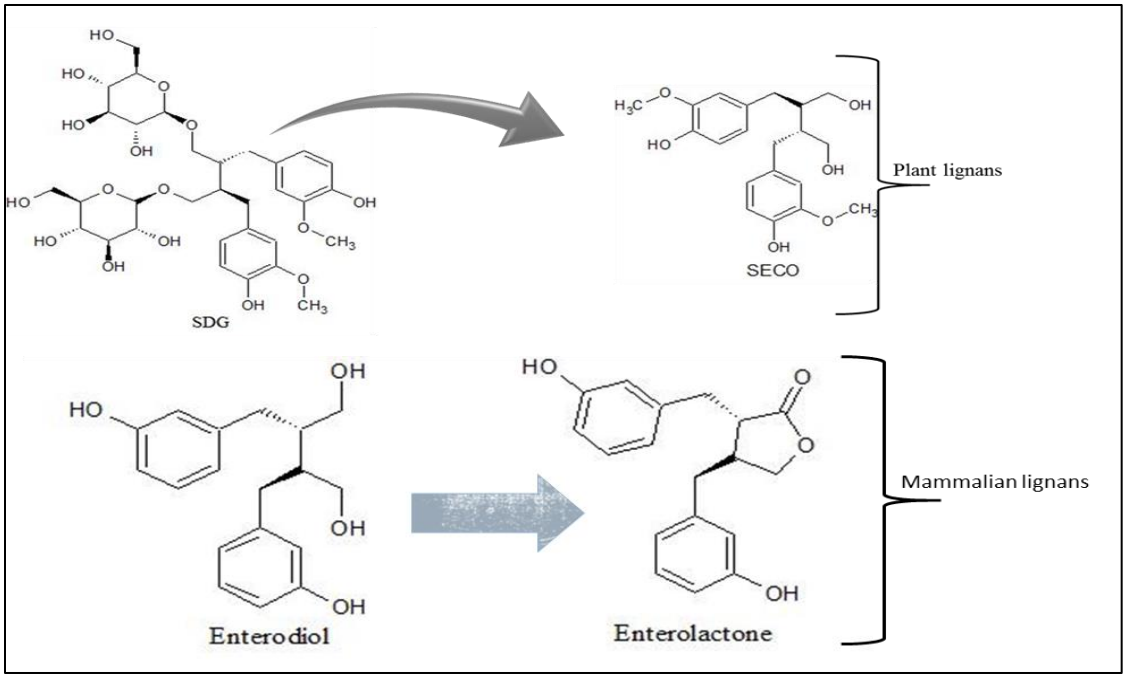

Figure 1.2 The transformation of SDG to SECO (plant lignans) are produced after the ingestion of the parent compound SDG initially from the oligomer that is further hydrolyzed to SECO. SECO in its turn is converted to the mammalian lignan END by the aid of intestinal microflora. END undergoes O-demethylation and dehydroxylation to form ENL.

In vitro studies performed in a Caco-2 cell monolayer demonstrated that END and ENL can be transported across the epithelial membrane via passive diffusion and extensively conjugated to glucuronide and sulfate (END>90% and ENL>99%) metabolites within 48 h[218, 219]. Finally, END and ENL show a very low bioavailability due to an extensive first-pass effect during absorption from the gastrointestinal tract[217].

In the systemic circulation, there is a lack of glycosidic conjugated molecules. This observation is not exclusive to SDG, it also extends to isoflavone lignans that are in their glycosidic forms, and their bioavailability is largely dependent on β-galactosidase intestinal content[220]. Therefore, it is strongly supported that SDG is converted into aglycone forms of lignans in the intestine before being absorbed. This is supported by recent findings where SDG is reported to be incapable of passively diffusing across the polarized intestinal epithelium of Caco-2 cell lines, unlike the lignans SECO, ENL, and END[221].

Microbial biotransformation of flaxseed bioactives is a prominent process in lignan absorption, where several types of microflora are involved in the production of those bioactives[222].

Bacteroides and *Clostridium* O-deglycosylate SDG to form SECO. In turn, SECO undergoes O-demethylation and dehydroxylation catalyzed by *Ruminococcus* products and *Eggerthella lenta*, respectively, to form END[223]. END, then, is dehydrogenated by *Lactonifactor longoviformis* to produce ENL[224]. Therefore, the processing of orally consumed flaxseed is susceptible to considerable inter-individual variation, not only because of different populations of intestinal microflora but also to the multiple steps of biotransformation involved.

A pharmacokinetic study in twelve healthy volunteers, where 1.31 μmol/kg dose of SDG was administered, showed that enterolignans (ENL and END) appeared in the plasma at around 8-10 hours. This study showed long residence time of ENL in comparison to END reaching up to 36 hours post purified SDG consumption[225]. Another pharmacokinetic study that was performed in male Wistar rats showed longer half-lives for SECO and END. This rat study failed to assess ENL due to mortalities at doses of 1 mg/kg IV and 10 mg/kg oral[217]. In addition, the previous study showed zero bioavailability for SDG and 25% bioavailability for SECO and less than 1% to END. This low bioavailability could be attributed to the extensive first-pass effect during absorption from the gastrointestinal tract[217].

1.2.6.1.2 Disposition (Distribution, Metabolism, and Excretion)

Flaxseed lignans are widely distributed to the whole body. ENL, in particular, can be detected in liver, lung, kidney, heart and brain in rats with higher distribution to the kidney, liver, and testes[226]. This extensive distribution might be attributed to their lipophilicity and inherent ability to bind to sex hormone binding globulin (SHBG)[227]. Typically, the distribution is high for both ENL and END, but when the dose is escalated a reduced conversion of END to ENL (saturation of oxidation) and an increased distribution for END is observed[228].

In vivo studies show that SECO is extensively metabolized into END and ENL with only a small portion of SECO detected in plasma or urine. In humans, this biotransformation process is found to take part mainly in the colon[229]. During the absorption phase SECO, END, and EL undergo significant conjugation with glucuronic acid and sulfate as phase II metabolic processes. Phase I metabolic reactions represent a minor metabolic process involving SECO and the mammalian lignans and these are characterized by oxidation and hydroxylation products of the lignans, detected using human, pig, rat and rhesus monkey microsomes[230, 231].

When compared through the intrinsic clearance parameter, the extent of hepatic microsomal glucuronidation was greater than that of intestinal activity[232]. Intestinally unconjugated ENL will

be delivered to the liver where it gets glucuronidated, then transported to the bile[233]. On the other hand, conjugated ENL is effluxed into the portal ciruclation through the MRP3 basolateral efflux transporter[234]. This activity will result in a prolonged repetitive introduction of ENL in the intestine through enteohepatic recycling. Enterohepatic recycling is reported for enterolignans in human, rats and pigs[216, 235]. In a human pharmacokinetic study, the appearance of a second peak after oral administration of purified SDG confirms that both ENL and END are susceptible to enterohepatic recirculation[235]. The involvement of the liver and intestine in glucuronidation in addition to enterohepatic recycling results in the availability of enterolignans in the intestine for a considerably favorable period of time and thus increasing exposure of the intestinal epithelium to the lignans.

Fecal excretion is the primary route of excretion of lignan metabolites, as 48% of an administered lignan dose is excreted fecally in pigs[236]. The fecal excretions of END, ENL and matairesinol conjugates in pre-menopausal women on normal diets were 80, 640 and 7.33 nmol/day, which is increased with flaxseed consumption up to 2560, 10300 and 11.9 nmol/day, respectively[237]. Lignan glucuronides are excreted in bile and subsequently undergo deglucuronidation by bacterial β-glucuronidase activity. The high fecal excretion of enterolignans may be due to incomplete absorption and enterohepatic circulation. This assumption is confirmed for another structurally related lignan (genestein), which was labeled with ^{14}C in intestinal location to confirm the involvement of enterohepatic circulation in the process of fecal excretion of glucuronidated lignans[238].

Urinary excretion is an important route for lignan metabolite excretion. Most of the ENL is excreted as ENL-glucuronide in urine while a minor amount is excreted as sulfates[239, 240]. END was more extensively excreted in urine when compared to ENL and SECO upon the administration of a standardized flaxseed supplement in women; this increase might be attributed to enhanced hydrophilicity of END, or potentially the higher plasma concentrations of END as a metabolite of flax lignan consumption[241].

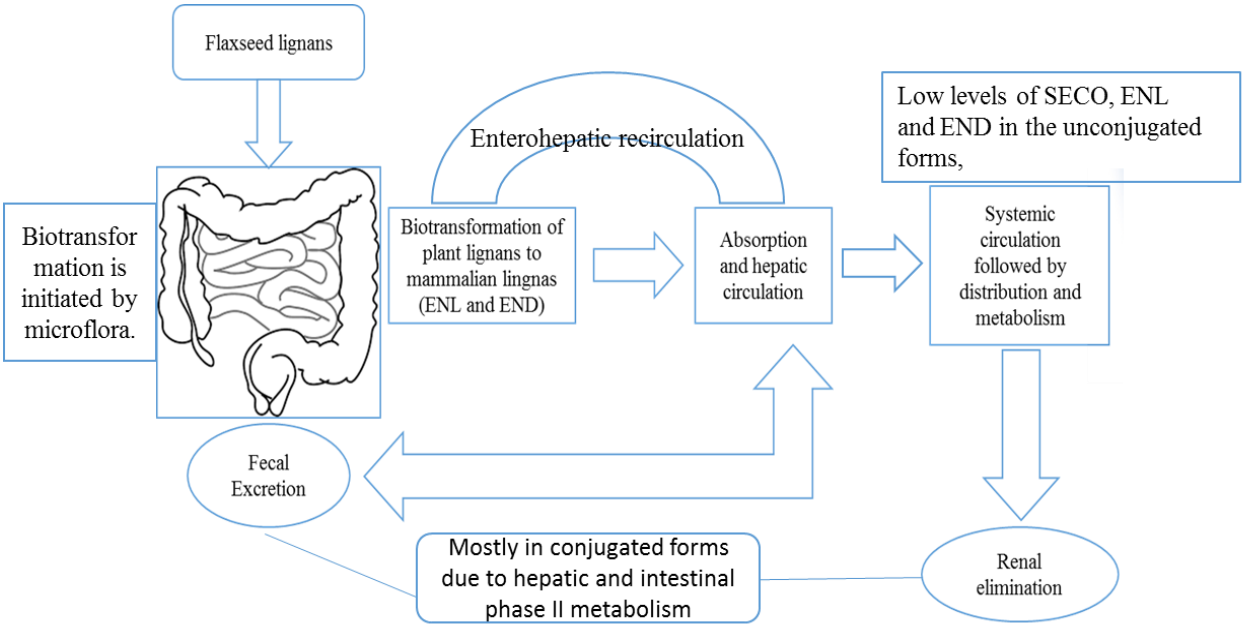

Figure 1.3 General PK scheme of lignans explains the potential hypothesis of initial biotransformation of SDG initially to END and finally to ENL by the aid of normal flora in the intestine, followed by absorption and further distribution and metabolism. Lignan susceptibility to enterohepatic circulation is fortified by the enzymatic activity in the intestine and the available microflora.

1.2.6.2 Linoorbitide (LOB) Pharmacokinetics

1.2.6.2.1 Chemistry of LOBs

Generally, cyclic peptides in plants are frequently abundant, and there are 455 cyclic peptides in *Caryophyllaceae* and *Rhamnaceae* plant families[242]. Chemical structure significantly influences the pharmacokinetics of any molecule; flax cyclic peptides are large in size with variable sequences of amino acids. Although flaxseed LOBs were discovered in 1959, LOBs were not extensively studied due to their structural complexity and stability issues that rendered extraction, isolation, and purification difficult and costly[32]. LOBs are a group of cyclic, hydrophobic peptides composed of eight or nine amino acid residues with molecular weights of approximately one thousand Daltons. The dominant amino acids in LOBs are (Phe), Leucine (Leu), Isoleucine (Ile), Methionine (Met) and Proline (Pro) amino acid residues as seen in table 1.3.

Table 1.3. Linoorbitides (LOBs) of flaxseed, amino acid sequences, and molecular weights [229-231].

Name	Amino acid sequence	Chemical formula and MWt (Da)
LOB-A	Ile-Leu-Val-Pro-Pro-Phe-Phe-Leu-Ile	$C_{57}H_{85}N_9O_9$ (1040)
LOB-B	Met-Leu-Ile-Pro-Pro-Phe-Phe-Val-Ile	$C_{56}H_{83}N_9O_9S$ (1058)
LOB-C	Mso-Leu-Ile-Pro-Pro-Phe-Phe-Val-Ile	$C_{56}H_{83}N_9O_{10}S$ (1074)
LOB-D	Mso-Leu-Leu-Pro-Phe-Phe-Trp-Ile	$C_{57}H_{77}N_9O_9S$ (1064)
LOB-E	Mso-Leu-Val-Phe-Pro-Leu-Phe-Ile	$C_{51}H_{77}N_8O_9S$ (977)
LOB-F	Mso-Leu-Mso-Pro-Phe-Phe-Trp-Val	$C_{55}H_{73}N_9O_{10}S_2$ (1084)
LOB-G	Mso-Leu-Mso-Pro-Phe-Phe-Trp-Ile	$C_{56}H_{75}N_9O_{10}S_2$ (1098)
LOB-H	Mso-Leu-Met-Pro-Phe-Phe-Trp-Ile	$C_{56}H_{75}N_9O_9S_2$ (1082)
LOB-I	Met-Leu-Mso-Pro-Phe-Phe-Trp-Val	$C_{55}H_{73}N_9O_9S_2$ (1068)
LOB-J	Msn-Leu-Val-Phe-Pro-Leu-Phe-Ile	$C_{51}H_{77}N_8O_{10}S$ (993)
LOB-K	Msn-Leu-Ile-Pro-Pro-Phe-Phe-Val-Ile	$C_{56}H_{83}N_9O_{11}S$ (1090)

Linoorbitide-A (LOB-A) was the first cyclic peptide to have its structure fully resolved in 1971 and fully identified by two-dimensional NMR analysis in 1995 (see figure 1.4)[14, 15, 243]. Conformational NMR studies concluded that LOB-A has no intramolecular hydrogen bonding. The analysis examined seven possible locations, five of which are exterior and two are located interior of the ring, and none was found to have hydrogen bonding[14].

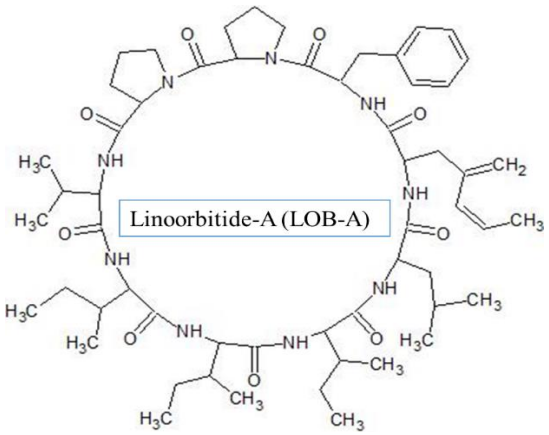

Figure 1. 4 LOB-A is the first cyclic peptide identified, composed of nine amino acids (Pro-Pro-Phe-Phe-Leu-Ile-Ile-Leu-Val) and has a molecular weight of 1040.3397 g/mol.

1.2.6.2.2 Pharmacokinetics of LOBs

Due to their recent discovery and the lack of extensive *in vivo* experimentation, LOBs have never been under the scope of pharmacokinetics analysis. To further understand the potential pharmacokinetic behavior of LOBs, information from other currently available cyclic peptides and their pharmacokinetic profiles as well as pharmaceutical principles can shed some light on possible LOB characteristics. In order to evaluate the potential of any chemical structure to behave as a drug-like molecule for oral administration, four rules were elaborated, commonly named Lipinski`s rule of five, to make *a priori* assessments of a molecule's drug likeness[244]. Lipinski's rules state that: a compound is more likely to be an orally available drug if it does not violate more than one of the following rules: 1) No more than five hydrogen bond donors. 2) No more than ten hydrogen bond acceptors. 3) An octanol: water partition coefficient (Log P) of less than 5, and 4) Molecular weight lower than 500 daltons [244]. According to these rules, LOBs are unlikely to exert any systemic pharmacological effects. The high molecular weight (~1000 daltons) and a high number of hydrogen bond donors and acceptors are unfavourable for oral absorption and, consequently, a failure to reach the systemic circulation is presumed.

In contrast to what is hypothesized by Lipinski`s rule of five, Cyclosporin-A (CsA) is a structurally relevant hydrophobic cyclic peptide that is capable of permeating from the gastrointestinal tract lumen to the systemic circulation. Studies in an *in vitro* transwell system of polarized of Caco-2 cells showed that CsA is capable of passively moving from apical to basal compartments, which indicates ability of CsA to permeate across the gastrointestinal mucosa. In addition, CsA permeation was also influenced by the addition of efflux transporter inhibitors (P-glycoprotein)[245]. Moreover, the bioavailability of CsA upon oral administration in humans reach as high as 29%[246, 247].

Taken together, both lignans and LOBs likely have low oral bioavailabilities. In addition to the significant enterohepatic recirculation of ENL and ED, lignans and LOBs will likely have high concentrations in the gastrointestinal lumen. Therefore, a significant need to assess their local effects on the intestinal epithelium is prudent to discover innovative utilization of their favorable pharmacological activity.

1.3 Rationale

Currently, three out of four Canadians frequently use natural health products, and one-third of them use natural products on a daily basis[248]. This inclination toward natural products has been the driving force for many governmental and industrial sectors to invest further in the regulation, production, and improved accessibility and customer confidence in natural products.

The reports on the effectiveness of flaxseed bioactives as anti-inflammatory agents and modulators of cholesterol levels, combined with the reported safety profile encourages further investigations with the flaxseed bioactives in several therapeutic areas. In addition, our pharmacokinetic knowledge about flaxseed bioactives suggests reduced systemic availability and high abundance at the level of the intestine. This assumption leads to their potential use as modulating biological activity at the level of the intestinal epithelium. Two specific pathologies, IBD and hypercholesterolemia, can benefit significantly from possible intestinal epithelial local effects. Both pathologies have need for improved treatment options as these conditions continue to frustrate both clinicians and scientists in their attempts to effectively mitigate disease pathology and significantly improve patient outcomes.

Based upon such considerations, flaxseed as a chemopreventative or chemotherapeutic agent might improve therapeutic outcomes in IBD and hypercholesterolemia patients. However, experimental support for the lignans and LOBs and their mechanisms of action is first required to support the use of flaxseed in these therapeutic areas.

1.4 Hypotheses

1- Enterolactone and Linoorbitides (LOBs) maintain intestinal epithelial barrier integrity through anti-inflammatory and anti-oxidative effects.

2- Inflammation increases pyruvate kinase M2 (PKM2) expression in intestinal epithelial cells and PKM2 is elevated in serum of newly diagnosed inflammatory bowel disease patients.

3- Enterolactone and enterolactone glucuronide modulate cholesterol metabolism in intestinal epithelial cells.

1.5 Objectives

1- To evaluate the efficiency of enterolactone (ENL) and linoorbitides (LOBs) to protect intestinal epithelial barrier integrity against an inflammatory stimulus, INF-γ and TNF-α, in cell lines simulating the intestinal epithelium.

2- Assessing the role of ENL and LOBs to modulate oxidative stress *in vitro* using an intestinal epithelial cell line and a co-culture model and the possible mechanisms that might be involved.

3- To determine whether newly diagnosed inflammatory bowel disease patients demonstrate elevated levels of circulating PKM2 and explore whether intestinal epithelial cells increase PKM2 expression following an inflammatory stimulus.

4- Investigate the capacity of ENL and ENL-glucuronide to modulate cholesterol metabolism in intestinal epithelial cells and the mechanism of action involved.

CHAPTER 2

Linoorbitides and enterolactone differentially ameliorate *in vitro* inflammation in HCT-8 intestinal epithelium

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Linoorbitides and enterolactone differentially ameliorate *in vitro* intestinal inflammation in HCT-8 intestinal epithelium.

Ahmed A. Almousa[1], Sharyle Fowler[2], Jennifer Jones[3], François Meurens[4,5]
Ed S. Krol[1], Jane Alcorn[1*]

Transitioning rationale:

The literature review provided a premise for the potential utilization of LOBs and ENL on the level of the intestinal epithelium. This utilization is favored in disease states that are significantly related to main functions of the intestinal epithelium, namely as a barrier and first line immune defense and as a significant player in cholesterol metabolism. This chapter aims to provide a proof of concept of the potential anti-inflammatory effects of ENL and LOBs to maintain intestinal epithelial barrier function. An *in vitro* transwell system to simulate intestinal epithelium served as an initial simple model to explore the maintenance of barrier integrity through assessments of trans-epithelial electrical resistance, Lucifer yellow rejection and the expression of ZO-1 tight junction proteins. Finally, this chapter explores initial screening of cytokines, chemokines and other inflammatory mediators involved to understand the mechanism involved in the anti-inflammatory effects of ENL and LOBs.

Contribution statement:

Ahmed Almousa contributed to this manuscript by designing the study, performing experiments, data acquisition, data analysis and manuscript drafting.

Abstract

Barrier integrity dysfunction is considered a hallmark of inflammatory bowel disease (IBD) pathogenesis, and its maintenance continues to be a target for drug discovery to ameliorate IBD. The current shift toward natural products to aid as adjuvant treatments in chronic diseases like IBD is driving the research for a better understanding of whether individual bioactives may positively modulate disease pathogenesis. This study investigated whether the flaxseed bioactives, Linoorbitides (LOBs) and Enterolactone (ENL), could maintain epithelial barrier integrity in an *in vitro* intestinal inflammation model. An inflammatory response was induced in HCT-8 polarized intestinal epithelia with the aid of TNF-α and INF-ɣ. The ability of LOBs and ENL to maintain barrier integrity was assessed through measurement of Trans-Epithelial Electrical Resistance (TEER), Lucifer Yellow rejection for barrier permeability, and assessment of zonula occludins 1 by immunohistochemistry. Results showed 200 nM of LOB-J, LOB-A, and ENL prevented inflammation-induced reduction in TEER with relative TEER values of 108.59%, 63.19% and 64.24%, respectively, at 24 hours. LOB-A and ENL caused a ~2- and 3.4-fold increase in PPAR-ɣ mRNA expression relative to untreated control, while LOB-A and LOB-J, but not ENL, significantly downregulated TNF-α expression. Western blot analysis confirmed gene expression results. Immunohistochemical analysis showed ENL, but not LOB-A and LOB-J, maintained ZO-1 expression relative to untreated, unstimulated control. The difference in magnitude, chronology, and nature of effect between these three flaxseed bioactives suggest their epithelial barrier protection is imparted through different mechanisms. Further investigations are warranted including evaluation in animal models of IBD.

2.1 Introduction

Inflammatory bowel disease (IBD) constitutes Ulcerative colitis (UC) and Crohn`s disease (CD) and is characterized by idiopathic chronic inflammation along the intestine with varying locations and clinical presentations[168]. IBD has unclear multifactorial etiology correlated to variable environmental, genetic, cellular, and immunological factors[104]. Intestinal epithelial barrier integrity is hypothesized to be distressed during the course of the disease and is considered a characteristic feature of IBD[249]. The presence of this barrier is partly granted by a set of integral cellular proteins, tight junction complexes and cell cytoskeleton microtubules and filaments, which maintain robust intercellular connections between the epithelial cells[119]. After impairment of intestinal barrier integrity, noxious molecules and pathogens leak to the lymphatic system to initiate a cascade of inflammatory reactions. Such inflammation is what leads to shedding of the mucosal epithelial layer as in UC or transmural inflammation of the intestinal mucosa as in CD[250], the hallmarks of IBD.

The mainstay of IBD treatment is corticosteroids. Chronic administration is associated with several side effects including weight gain, gastric irritation, and potential loss of bone density and increased risk of fracture[251, 252]. In addition to corticosteroids, immunosuppressants and biologicals leave patients more susceptible to viral and bacterial infections[253]. In conjunction with pharmacological agents, many patients lean toward using natural products to aid the healing and remission of active IBD flares. Studies evaluating the use of natural products by IBD patients report benefit from their use[254]. Among those natural products used is flaxseed, which is reported to ameliorate inflammation in general, in addition to its potential role in hypercholesterolemia and cancer[4, 5, 12].

Flaxseed contains high levels of the bioactive compounds, lignans and linoorbitides (LOBs)[29, 255]. Following an oral administration of flaxseed extracts, the plant lignan, secoisolariciresinol diglucoside, undergoes conversion to its aglycone, secoisolariciresinol, with subsequent biotransformation to the mammalian lignan, enterodiol, and then to enterolactone (ENL) by the metabolic activity of intestinal microbiota[256]. The mammalian lignans have low oral bioavailability and are susceptibile to enterohepatic circulation[233]. This aforementioned factor favors the potential for enhanced localized pharmacological activity in the intestine[238, 257]. Although the biological activity ascribed to the different lignan metabolites is incompletely known, the literature identifies ENL as one of the biologically active metabolites[44, 96, 257]. The linoorbitides,

LOB-A and LOB-J, are cyclic peptides composed of nine and eight amino acids and have molecular weights of 1040 Da and 993 Da, respectively [15, 258]. Their large molecular weight and hydrophilic nature hinders their permeation through the intestinal epithelium. Lack of absorption due to these reasons, in addition to reported immunosuppressant activity, suggest the potential utilization of linoorbitides to ameliorate intestinal inflammation locally. Collectively, this research aims to assess the potential effect of the flaxseed bioactives, linoorbitides and enterolactone, on intestinal epithelium integrity in an *in vitro* intestinal inflammation model, confirm their protective effects, and reveal potential mechanisms of action.

2.2 Materials and Methods

2.2.1 Cell culture

The HCT-8 cell line was purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA) and maintained in RPMI media (ATCC) supplemented with 10% horse serum and 1% penicillin/streptomycin (10,000 U, 10 m/mL) from Sigma-Aldrich (St. Louis, MA, USA). All other materials were bought from Sigma-Aldrich unless otherwise mentioned. Initially, cells were passaged twice after removal from liquid nitrogen vapour with media changes every two days. Cells grown in Corning™ T-75 flasks (Pittsburgh, PA, USA) were passaged at 70-80% confluency using 0.25% Trypsin-EDTA. Cells were constantly maintained at 37°C and 5% CO_2. All experiments were conducted with cells at passage number 4-15.

2.2.2 Cytotoxicity assays

Cytotoxicity assays of LOBs and ENL utilized the Calcein-AM kit from Biotium (Houston, TX, USA). Cells were seeded in 96-well plates at a density of 10,000 cells/well within the calibration range of the provided kit. Cells were allowed to attach for 24 hours, the media aspirated, and new media containing LOB-A, LOB-J and ENL in the range of 25 nM- 200 µM or positive control of 4 mM 5-FU was added to the cells. Cells were exposed for 72 hours to the compounds and subsequently washed with Calcein washing buffer, followed by addition of Calcein working solution and incubated for 30 minutes at 37°C and 5% CO_2. Cell viability was determined using a fluorescence plate reader taken at excitation and emission wavelengths of 495 and 515 nm, respectively, using a Biotek Synergy HT microplate reader (Fisher Scientific, Canada).

2.2.3 Induction of inflammation in HCT-8 monolayer

HCT-8 cells in RPMI supplemented media were plated on Corning™ Transwell (0.4 μM, 24-well plates; Fischer Scientific, Pittsburgh, PA, USA) at an average seeding density of 5×10^4 cells per insert. The cells were allowed to polarize for 8 days to a minimum Trans-Epithelial Electrical Resistance (TEER) of 2000 ohms/cm^2, and media was replaced every other day (200 μL on the apical side and 600 μL on the basal side) following vacuum suction of the old media. TEER values were measured using Millicell ERS™ voltohmeter by EMD Millipore® (Billerica, MA, USA) after 30 minutes of acclimatisation in a biosafety cabinet. At the time of inflammation induction, supplemented media was removed and substituted with Hank's balanced salt solution (HBSS) (no calcium) combined with TNF-α (10 ng/mL) and INF-γ (50 ng/mL) added to the basal compartment of transwells and media in the apical compartment. The final concentration of cytokines used to elicit an inflammatory response was determined by optimization experiments with measurement of TEER changes. The flaxseed bioactives (LOB-A, LOB-J, and ENL) were added simultaneously with the cytokines in four replicates and at four different concentrations on three different experimental days to both compartments. The bioactives were initially dissolved in 100% dimethylsulfoxide (DMSO) from ATCC, and then serially diluted to 1% DMSO with horse serum free RPMI media and used as a final working solution. TEER measurement was done at the beginning of treatment, and at 24 and 48 hours after exposure. LOB-A and LOB–J were kindly gifted from Prairie Tide Chemicals Inc. (Saskatoon, Canada).

2.2.4 Lucifer yellow rejection

After treating transwells as explained above for 48 hours, lucifer yellow (LY) was introduced to the apical (200 μL) compartment at a 100 μg/mL concentration and incubated for two hours at 37°C and 5% CO_2. On the same plate the no-insert wells were either supplied with 800 μL of HBSS at 100% epithelial integrity or 600 μL HBSS + 200 μL LY to represent 100% leakage of epithelium. Rejection rates were obtained by reading fluorescence at 485/535 nm in the basal compartment using a Biotek Synergy HT microplate reader. Rejection percentages were calculated to correspond to epithelial integrity. Rejection rates were calculated by subtracting 100% Integral wells (HBSS) values from treated wells and 100% leakage wells, then dividing treated over 100% leakage, with subtraction of the result from 100%. Optimization experiments

which were guided by TEER results identified the appropriate concentrations used for these experiments.

2.2.5 Zonula Occludins 1 (ZO-1) immunohistochemistry

HCT-8 cells were grown on six well plates with glass cover slips and allowed to grow to 90% confluency to better simulate a monolayer epithelium. Cells were then treated with TNF-α (10 ng/mL) and INF-γ (50 ng/mL) alone or with flaxseed (LOB-A, LOB-J, and ENL) or 1% DMSO alone. After 24 hours of treatment cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature. The cells were subsequently blocked in 5% bovine serum albumin (BSA) and 0.5% Triton-X 100 for 3 hours at room temperature before incubation with primary antibody ZO-1 (ab59720) from Abcam (Toronto, ON, Canada) at (1:100) in blocking solution for 4 hours at 37°C and 5% CO_2. Goat anti-rabbit IgG (H+L) secondary antibody (Alexa Fluor® 488 conjugate) was used as the secondary antibody purchased from Thermo-Fisher Scientific (Waltham, MA USA). Visualization took place under a ZOE fluorescence microscope (Bio-Rad, Hercules, CA, USA). Optical density measurement for ZO-1 fluorescence used ImageJ software by National Institute of Health[259].

2.2.6 Extraction of total RNA and quantitative RT-PCR (qPCR)

After induction of inflammation for 24 hours, total RNA was extracted from cells cultured on transwells by using Qiagen RNeasy mini kit and Trizol (Toronto, ON, Canada) by following the manufacturer protocols. A Nanoview UV spectrophotomer (GE Healthcare Life Sciences, Quebec, Canada) was used to assess the concentration and purity of total RNA by measuring absorbance at 260 nm and the absorbance ratio (A_{260} / A_{280}) of a diluted sample of RNA, respectively. Al1 samples used for qPCR were of high purity and total RNA was stored at -80°C until further processing.

Total RNA was reverse transcribed Multiscribe reverse transcriptase to cDNA, and then gene expression quantified using Power Sybr® Greeen PCR Mastermix, all purchased from by Life Technologies (Burlington, ON, Canada). The polymerase chain reaction was carried out using a one-step method. Initial incubation with reverse transcriptase took place at 42ºC for 30 minutes, followed by denaturation at 95ºC for 3 minutes, 40 cycles of annealing and extension performed for 15 seconds and 30 seconds, respectively, and eventually a dissociation curve as per manufacturer's setting. This experiment was executed in an Applied Biosystems® 7300 system

(Foster City, CA, USA). Primer sequences using Integrated DNA Technologies (IDT) Primer-Quest tool (https://www.idtdna.com/Primerquest/Home/Index) for target genes were: 5`-CTCAAACGAGAGTCAGCCTTTA-3` (forward) and 5`-GTGGGAGTGGTCTTCCATTAC-3` (reverse) for PPAR-γ, 5`-GCCTGTAGCCCATGTTGTAG-3` (forward) and 5`-AGGACCTGGGAGTAGATGAG-3` (reverse) for TNF-α, 5`-TGGGAACAAGAGGGCATCG-3` (forward) and 5`-CCACCACTGCATCAAATTCATG-3` (reverse) for succinate dehydrogenase complex subunit A (SDHA). All primers were purchased through IDT (Iowa, USA). SDHA served as a housekeeping gene to normalize Ct values and relative quantitation was done using the delta-delta Ct method ($2^{-\Delta\Delta CT)}$.

2.2.7 Western blots of TNF-α and PPAR-γ

HCT-8 cells were seeded in T-25 flasks at a density of 1×10^6 cells/mL, and left to attach for 24 hours. Treatment with various concentrations of LOB-A, LOB-J, and ENL was carried out in addition to an inflammatory stimulus of TNF-α and INF-γ (10 and 50 ng/mL, respectively) for another 24 hours. Cells were lysed for protein extraction using radioimmunoprecipitation assay buffer (RIPA) from Millipore (Billerica, MA, USA) in ice-cold conditions. Protein concentrations were determined using the Pierce™ BCA Protein Assay Kit (Rockford, IL, USA), where linearity of protein concentrations was verified. Twenty micrograms of total protein were transferred for gel electrophoresis (Bio-Rad Laboratories). Membrane blocking was carried out using 5% BSA in 1X phosphate buffered saline Tween-20 (PBST) for one hour. All primary antibodies (1:500) were incubated overnight at 4°C in PBST and then incubated with the secondary antibody (1:2000) for 2 hours at room temperature. The blots were visualized using enhanced chemiluminescence detection system (Novex® ECL) from Life Technologies (Burlington, ON, Canada). PPAR-γ antibody (sc-7273) and β-actin antibody (sc-47778) were purchased from Santa Cruz Biotechnology (Dallas, TX, US). TNF-α antibody (ab1793) was purchased from Abcam (Toronto, ON, Canada). Fab2-Goat anti-Mouse IgG (H+L) Secondary Antibody-HRP conjugate (A24512) was purchased from Thermo-Fisher Scientific.

2.2.8 Statistical Analysis

Effects of treatments relative to controls were evaluated by use of one-way ANOVA followed by Dunnett's test to compare with controls. Differences were considered significant at *p*-

value < 0.05. All data analysis was performed by using GraphPad Prism 5 software (San Diego, CA, USA).

2.3 Results

2.3.1 Epithelial barrier protection by linoorbitides, enterolactone, and their combination

Cytotoxicity assay results showed that LOB-A is the most cytotoxic, followed by LOB-J and ENL was the least cytotoxic. These evaluations guided the concentrations used in all subsequent experiments. HCT-8 cells were used to simulate the intestinal epithelium monolayer to allow assessment of the ability of the flaxseed bioactives to maintain epithelial barrier integrity during an inflammatory stimulus. Relative to untreated control transwells, LOB-A, LOB-J, ENL, and their combinations generally maintained higher TEER values compared to time zero as seen in Figure 2.1 and 2.2. The usage of TNF-α and INF-γ to induce inflammation resulted in a fairly consistent reduction in TEER values (TEER ~30%) in untreated controls. At 24 hours, LOB-J reduced the degree of TEER reduction following an inflammatory stimulus in a concentration-dependent fashion such that at the highest concentration no reduction in TEER in HCT-8 polarized epithelium was observed. At 48 h, only the highest concentration of LOB-J resulted in statistically higher TEER values relative to untreated controls. In general, LOB-A at all concentrations maintained TEER values between 63.2 – 76.5% at 24 h, which was higher than untreated controls (27.5%), and between 37.1 – 48.2% 48 h, which was greater than untreated controls (23.1%). The highest concentration of ENL resulted in significantly higher TEER values, 64.2% and 56.04%, relative to untreated controls at 24 and 48 hours (31.3% and 25.5%), respectively. With the combination of LOBs and ENL, the relative TEER values compared to untreated controls were significantly higher at 24 h with a similar pattern, but with lower TEER values, emerging at 48 h. The highest relative TEER values were observed with the combination of ENL with LOB-J, while the lowest TEER values were observed with the combination of LOB-J and LOB-A.

2.3.2 Lucifer yellow rejection

To provide further confirmation of the maintenance of barrier integrity, we assessed Lucifer yellow rejection rate as a marker of paracellular transfer across the HCT-8 epithelium. Only the most effective concentrations of LOB-A, LOB-J, and ENL in the TEER experiments were assessed with the lucifer yellow rejection assay. Results at 48 hours in the transwell system showed that lucifer yellow rejection values were significantly higher with ENL (99.6%) and LOB-

J (98.8%), while LOB-A (96.9%) had similar rejection rates to untreated control (97.3%) (Figure 2.3).

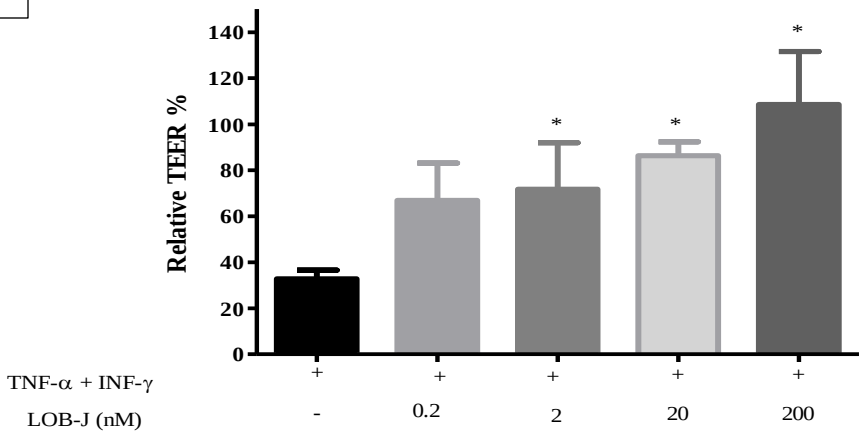
2.3.3 Immunohistochemistry of Zonula Occludens-1 (ZO-1)

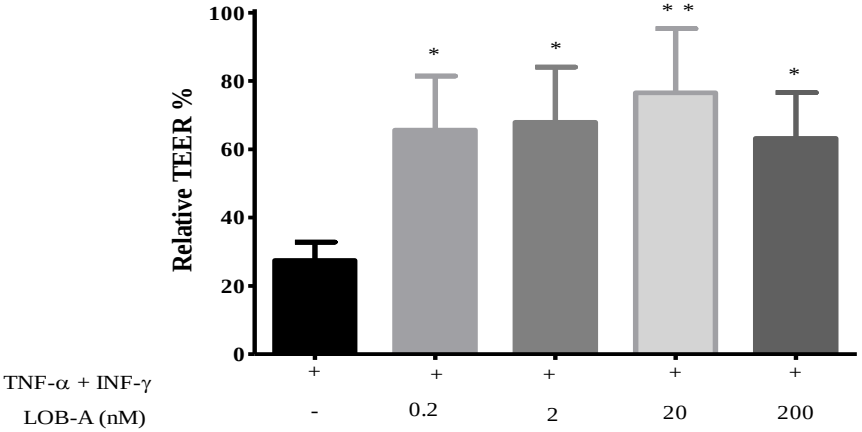
Tight junctions represented by ZO-1 is considered a cornerstone of barrier integrity and barrier function. To investigate the effect of LOB-A, LOB-J, and ENL on epithelial membrane integrity after an inflammatory insult, we examined ZO-1 protein expression in HCT-8 cell lines during the inflammatory insult for 48 hours with and without the flaxseed bioactives. Immunohistochemistry results showed that the inflammatory stimulus reduced ZO-1 expression in HCT-8 approximately 2-fold relative to untreated, unstimulated control (Figure 2.4). Treatment with 200 nM ENL maintained ZO-1 expression relative to untreated, unstimulated control, but with exposure to LOB-A and LOB-J ZO-1 expression was significantly decreased to levels consistent with untreated HCT-8 cells exposed to an inflammatory stimulus.

2.3.4 Up-regulation of PPAR-γ and down-regulation of TNF-α

To evaluate other potential mechanisms involved in the protective effect of flaxseed bioactives, expression of several innate immune response genes was evaluated. No significant changes in IL-1β, IL-6, IL-8, CCL-20 and MUC-2 gene expression was noted (data not shown). For TNF-α and PPAR-γ results from qPCR analysis showed that the acute phase reactant protein TNF-α was downregulated by both 0.2 nM LOB-A and 2 nM LOB-J at 2.23 fold reduction, while ENL at 200 nM caused a nonsignificant reduction in TNF-α mRNA expression (Figure 2.5). High concentrations (200 nM) of both ENL and LOB-A caused a 2-fold and 3.5-fold increase in PPAR-γ mRNA expression, respectively. Western blots confirmed the changes in mRNA expression for the respective treatments (Figure 2.5).

A


Relative TEER %
140
120
100
80
60
40
20
0
*
*
*
TNF-α + INF-γ
LOB-J (nM)
+ + + + +
- 0.2 2 20 200


Relative TEER %
100
80
60
40
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0
*
*
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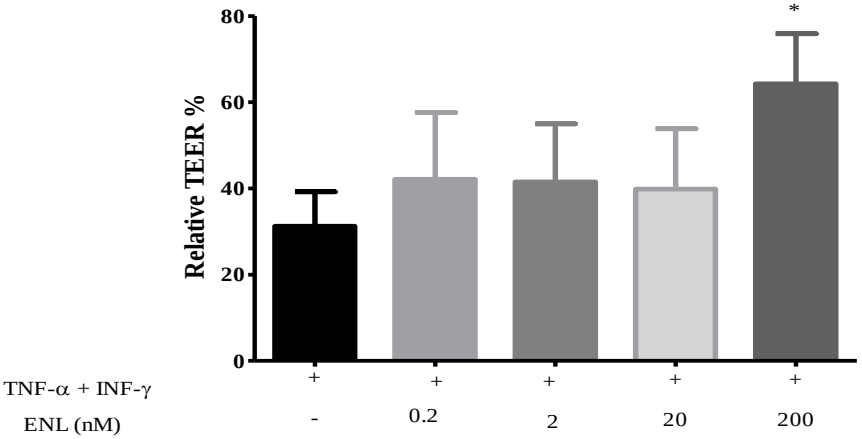
*
TNF-α + INF-γ
LOB-A (nM)
+ + + + +
- 0.2 2 20 200


Relative TEER %
80
60
40
20
0
*
TNF-α + INF-γ
ENL (nM)
+ + + + +
- 0.2 2 20 200

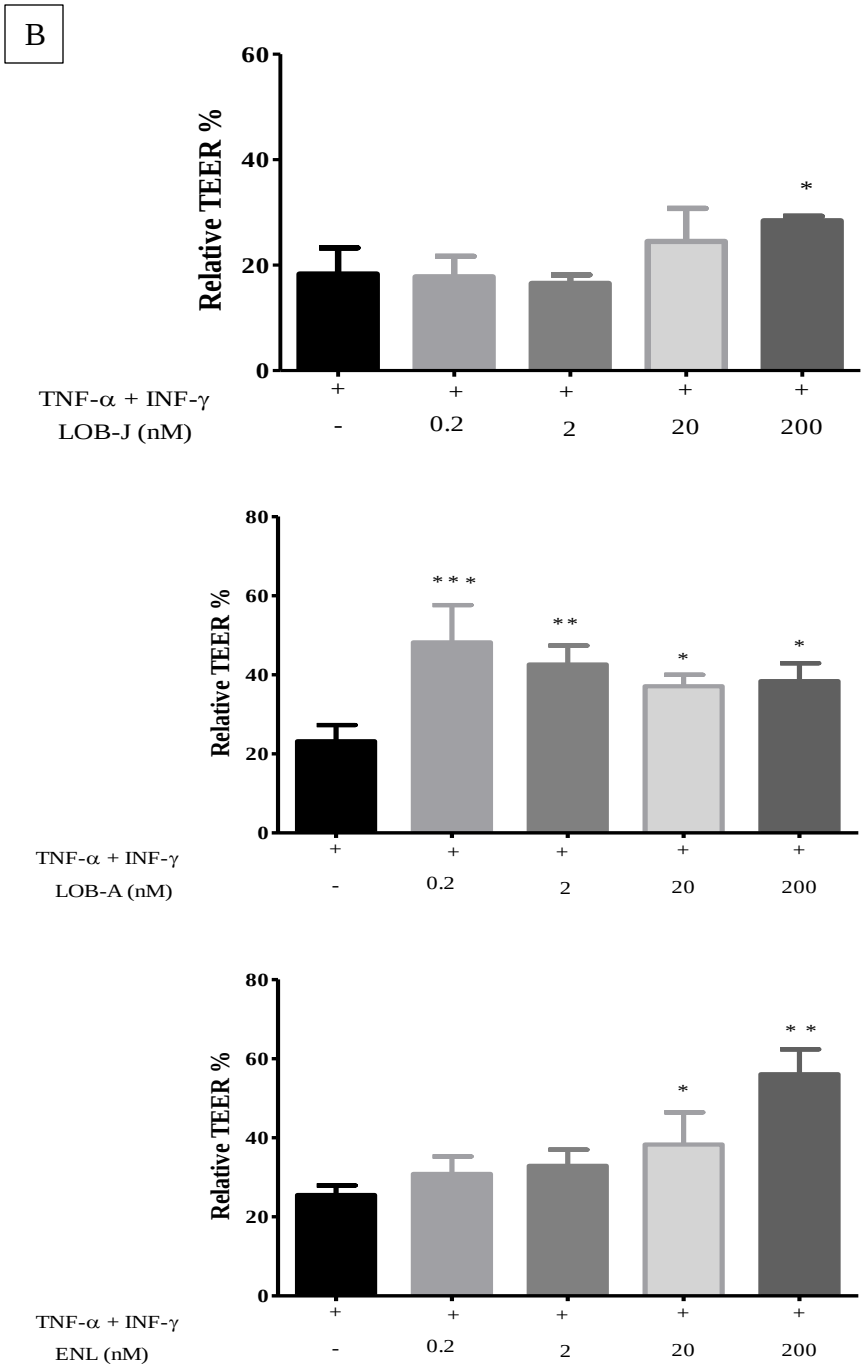


Figure 2.1 Effect of LOB-J, LOB-A and ENL on HCT-8 epithelial monolayer transepithelial electrical resistance (TEER) upon inflammatory insult with 10 ng/mL TNF-α and 50 ng/mL INF-γ for 24 hours (A) and 48 hours (B). Results represent TEER values at 24 or 48 hours relative to TEER at time zero. Data are presented as means ± S.D of 4 replicates determined on 4 different occasions. * p-value < 0.05, ** p-value < 0.01, *** p- value < 0.001.

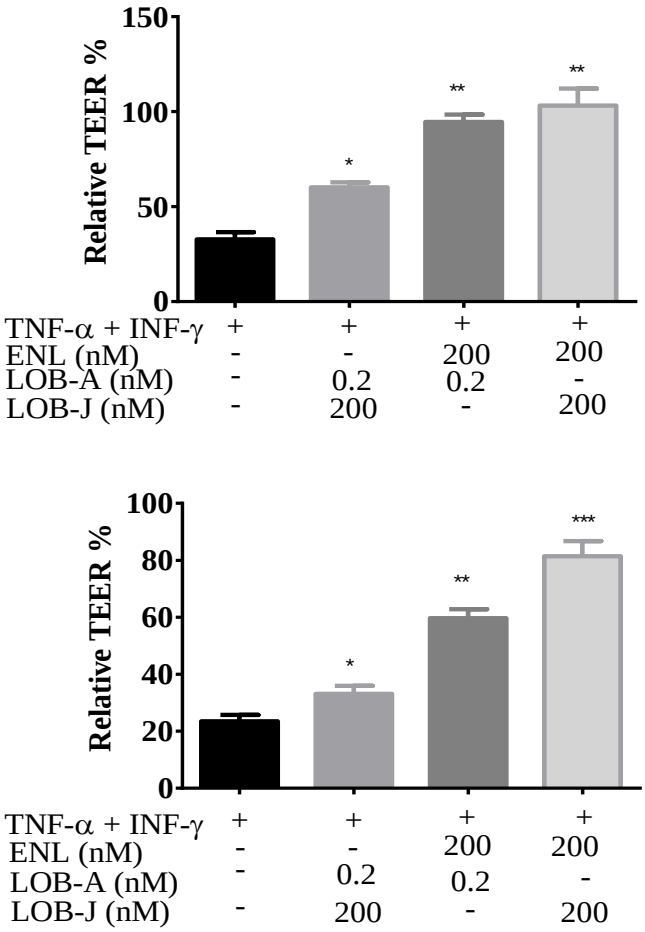


Figure 2.2 Effect of combining LOB-A, LOB-J and ENL on transepithelial electrical resistance (TEER) in the HCT-8 epithelial monolayer upon inflammatory insult with 10 ng/mL TNF-α and 50 ng/mL INF-γ) for 24 hours (A) and 48 hours (B). ENL in combination with LOB-J had the highest TEER values at both 24 and 48 hours. Results represent TEER values at 24 and 48 h relative to TEER at time zero. Data are presented as means ± S.D of three replicates on three separate occasions. * p-value < 0.05, ** p-value < 0.01, *** p- value < 0.001.

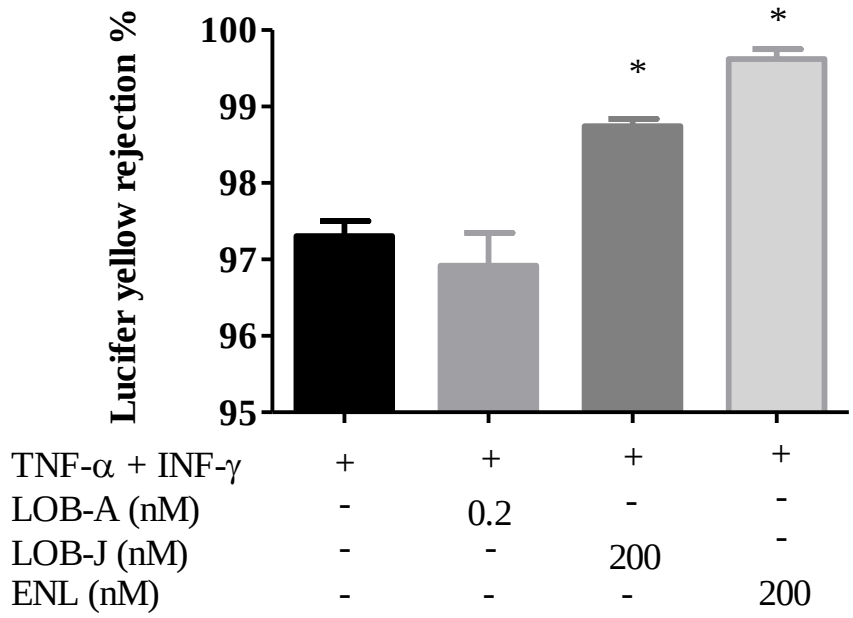
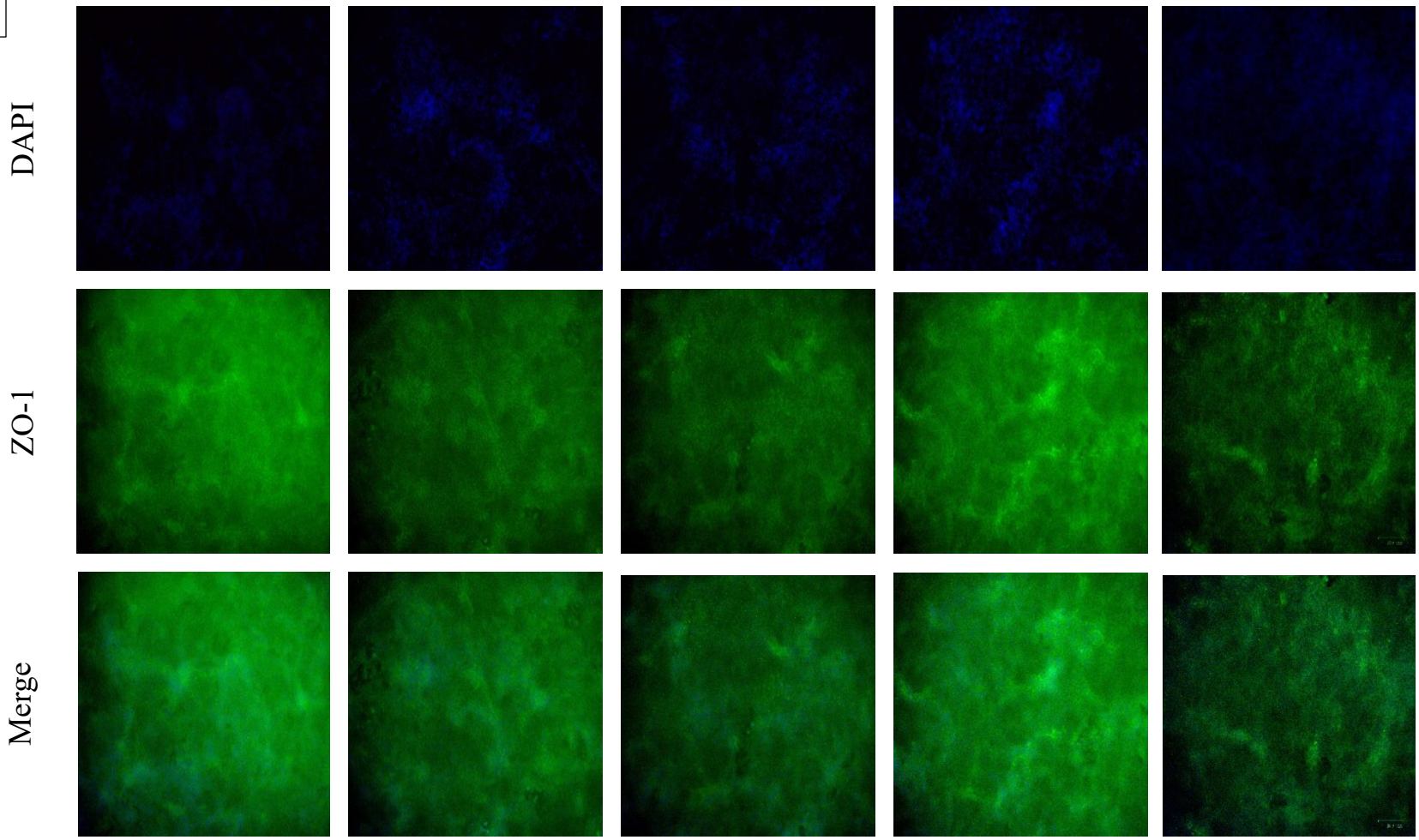


Figure 2.3 Lucifer yellow (LY) rejection after 48 hours incubation with LOB-A, LOB-J, and ENL. Data are presented as means ± S.D of three replicates on three separate occasions. * p-value < 0.05.

A


DAPI
ZO-1
Merge
Vehicle control (1 % DMSO)
0.2 nM LOB-A + TNF-α + INF-γ
200 nM LOB-J + TNF-α + INF-γ
200 nM ENL + TNF-α +INF-γ
TNF-α + INF-γ

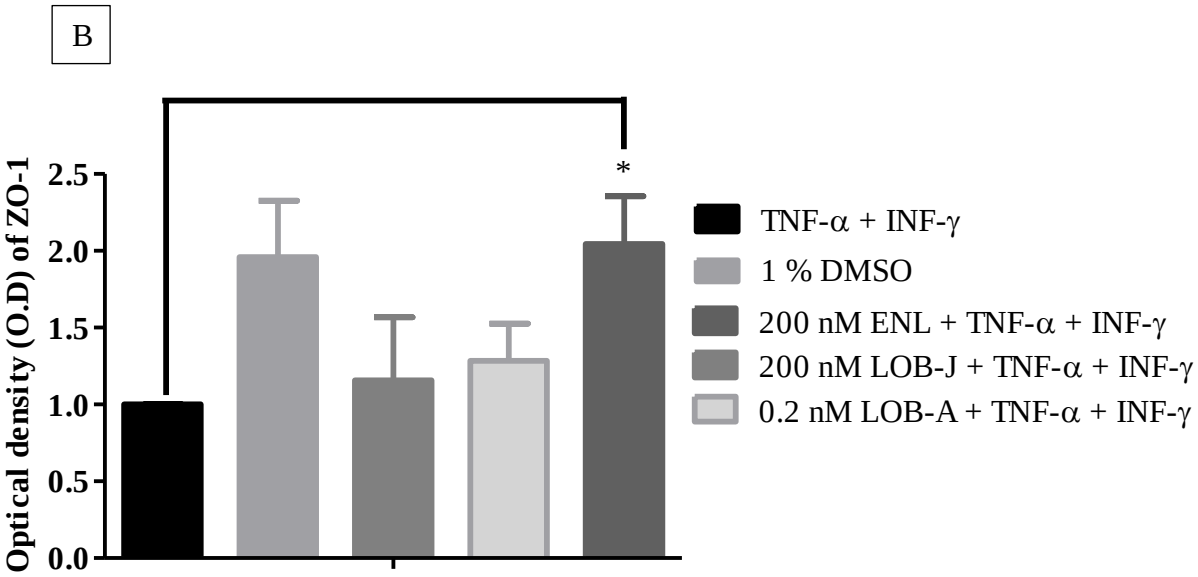


Figure 2.4 Immunohistochemistry of zonula occludens 1 (ZO-1) in HCT-8 epithelial monolayer (48 hours treatment). A: Immunofluorescence images showing loss of ZO-1 intensity upon induction of inflammation with 10 ng/mL TNF-α and 50 ng/mL INF-γ relative to control (1% DMSO). B: Optical density measurement for ZO-1 fluorescence using ImageJ software. Data are presented as means ± S.D of three replicates on three separate occasions. * p-value < 0.05.

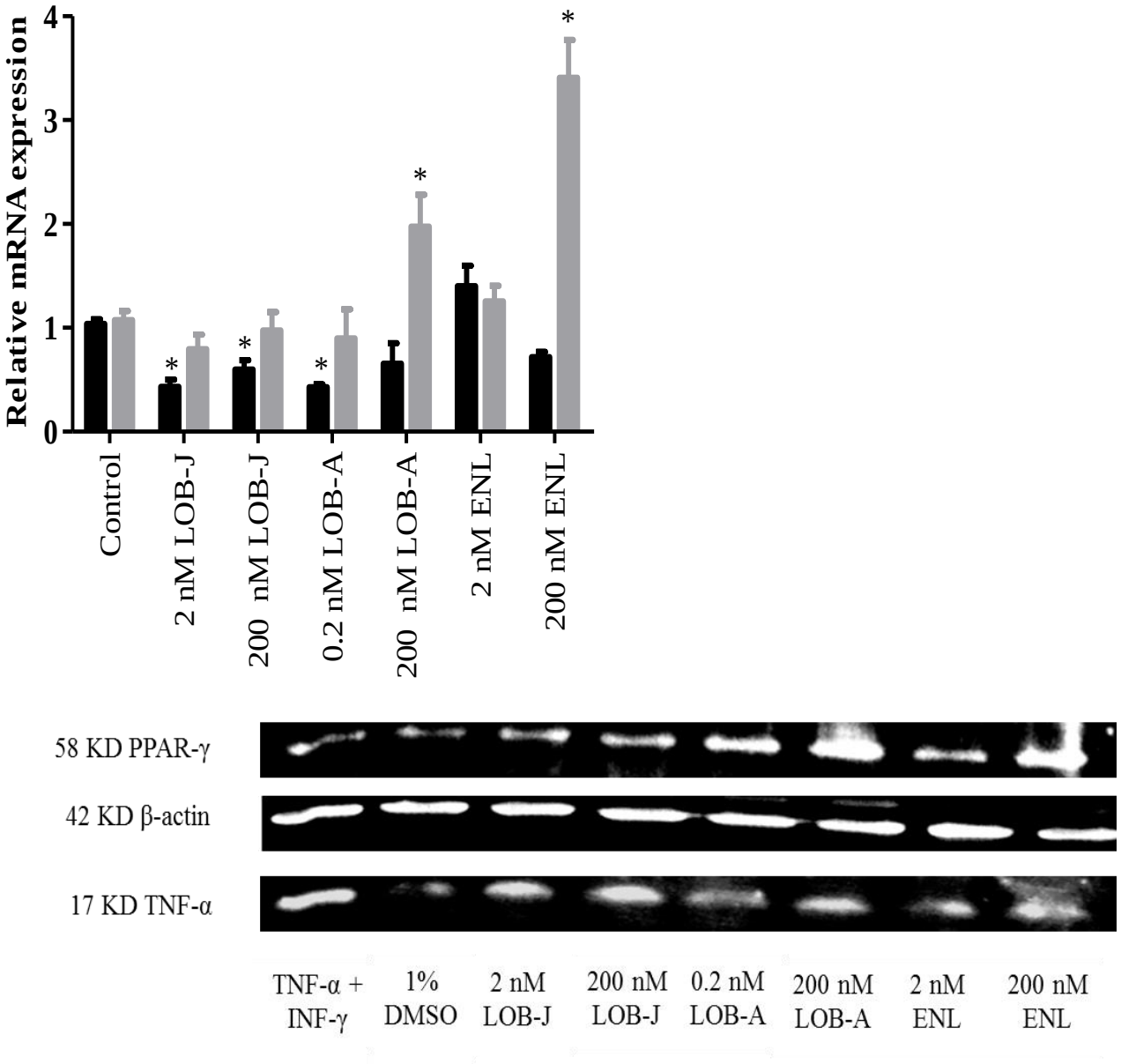


Figure 2.5 Relative mRNA expression and western blots for TNF-α (solid black bars) and PPAR-γ (light gray bars). Data are presented as means ± S.D of three replicates on three different occasions. * p-value < 0.05.

2.4 Discussion

Effective management of IBD remains a significant challenge to both the health system and to patient quality of life[186, 187, 253]. Although the biologics have improved treatment efficacy, their use has shifted health care expenditures to the actual cost of the medication[260]. A solution to these escalating costs may be the development of cost-effective adjuvant therapies that help improve efficacy of less expensive existing treatments. Natural products with anti-inflammatory and immunomodulatory activities are purported alternatives to IBD treatment and management[254]. As natural products, flaxseed bioactives, cyclic peptide Linoorbitides (LOBs) and lignans, have evidence of ability to mitigate inflammation and oxidative stress or elicit immune modulating effects[8, 96]. Such properties have led researchers to evaluate these flaxseed bioactive compounds in other chronic disease states[3, 82, 85], but very few studies report on their effects in IBD[17,9]. In the present study we selected flaxseed LOB-A, LOB-J, and the mammalian lignan, ENL, based on their known physicochemical and pharmacokinetic properties to assess their potential use to protect against intestinal epithelial barrier dysfunction[9, 51, 261, 46]. These flaxseed bioactives imparted protective effects on intestinal epithelial barrier function *in vitro* variably involving anti-inflammatory, antioxidant, and/or immunosuppressive effects.

Chronic inflammation and defective intestinal epithelial barrier integrity are hallmarks of IBD[119]. The pro-inflammatory cytokines, TNF-α and INF-γ, have been shown to disrupt barrier integrity in both *ex vivo* and *in vitro* studies of the intestinal epithelium[262, 263]. Our study involving the HCT-8 polarized epithelium is consistent with these studies, where we observed a time-dependent and significant reduction in TEER and LY rejection rates with exposure to TNF-α and INF-γ. Although the HCT-8 cell line is not frequently used to simulate the intestinal epithelium monolayer, its ability to form a strong barrier with a high transepithelial resistance in a short time (8 days) favoured its usage over the Caco-2 cell line. The reduction in TEER and LY rejection rates with TNF-α and INF-γ stimulation supported its use to assess the protective effect of the flaxseed bioactives. Both cytokines were necessary as TNF-α or lipopolysaccharide alone was not sufficient to cause significant reductions in TEER consistent with other studies employing the Caco-2 cell line[263]. In the HCT-8 polarized epithelium LOB-A, LOB-J, ENL, and their combinations generally maintained higher TEER values suggesting a protective effect of the LOBs and ENL against inflammation-induced loss of barrier integrity. Although Zarepoor *et al.*[18] reported that dietary flaxseed intensified the colonic mucosal damage in a mouse model of IBD,

the lignan enriched hull fraction of flaxseed did not exacerbate colitis, possibly lending support for use of lignans and LOBs in the absence of the oil fraction of flaxseed to mediate protective effects on the colonic mucosal barrier *in vivo*. These observations are aligned with literature reports on the effects of LOBs and ENL with regards to their anti-inflammatory and immunosuppressant effects[8, 10].

Interestingly, the protective effect of the LOBs and ENL exhibited differences in both magnitude and chronology suggesting the involvement of different mechanisms of action. The tight junction complex plays an integral role in maintenance of barrier function and zonula occludin 1 (ZO-1) is principally responsible for tight junction complex assembly[264]. Therefore, our assessment focused on ZO-1 protein and its subsequent morphological effect on maintaining barrier integrity, high trans-epithelial electrical resistance (TEER), and enhanced lucifer yellow (LY) rejection. Our results showed that an insult to HCT-8 with TNF-α and INF-γ reduced the overall expression of ZO-1, reduced TEER, and promoted leakage of LY. The mammalian lignan, ENL, mitigated downregulation of ZO-1 expression at 48 h, while ability of low concentration LOB-A to maintain ZO-1 expression was lost. With ENL, the favourable and enhanced concentration-response effect on TEER and LY rejection values at 48 hours, though, suggests a possible role for an ENL metabolite in protection of epithelial integrity since previous studies indicate complete glucuronidation and sulfation of ENL within 24 hours of incubation in intestinal epithelial cells[219, 221]. Alternatively, ENL is known to influence several transcription factors including NF-κB and, thus, its favourable effects can be delayed[44]. For the LOBs the loss in ability to maintain barrier integrity by 48 hours was associated with low expression of ZO-1. The LOBs antioxidant activity might be lost over time due to depletion of scavenging capacity. The potential for degradation is also a plausible explanation; however, when LOB-J was exposed to harsh thermal and oxidative stability testing it was resistant to chemical degradation and highly stable among other LOBs[258]. Interestingly, LOB-A showed a reverse concentration-response at 48 hours and minimal protection for the highest concentration at 24 hours. This suggests the involvement of significant cytotoxic effects of LOB-A at higher concentrations where barrier integrity is altered and apoptosis is potentially induced[33].

The difference in chronological effects between ENL and LOBs might also reflect differences in their ability to regulate PPAR-γ and the key inflammatory cytokine, TNF-α. ENL, and to a lesser extent high concentrations of LOB-A, caused sustained increases in PPAR-γ

expression, which seemed to relate to preservation of tight junction protein ZO-1 expression. On the other hand, LOB-J had short lived protection that seemed to be triggered by a greater reduction in the acute inflammatory mediator, TNF-α, consistent with its anti-inflammatory and anti-oxidant activity.

Significant downregulation of PPAR-γ is associated with IBD in murine models [265] and humans with UC[266]. This is mainly attributed to the role of PPAR-γ in trans-repressing signal dependent NF-κB activity[267], and further suppression of cytokines and chemokines involved in aggravating inflammation on the intestinal epithelium. Targeting PPAR-γ to ameliorate IBD has been attempted through PPAR-γ gene delivery [268] and use of the PPAR-γ agonists, such as rosiglitazone [269], which has been clinically shown to effectively treat mild to moderate UC in humans[270]. Similarly, we anticipate that the increased expression of PPAR-γ associated with ENL and LOB-A may positively reduce inflammation in IBD in general and likely with greater benefit in UC patients. This can be further applied to investigate the optimal composition of these components to reduce intestinal inflammation.

In summary, ENL, LOB-A and LOB-J alone or in combination mitigated the loss of *in vitro* barrier integrity with an inflammatory stimulus encompassed by effects on TEER values, LY and ZO-1. Screening for genes involved in this protection showed that reduction of TNF-α expression by LOBs and ENL and increased PPAR-γ expression by LOB-A and ENL. This systematic evaluation of flaxseed ENL and LOBs is suggestive of different mechanisms of action for each individual bioactive, suggesting that their combination in appropriate ratios may result in improved protection of epithelial barrier. Further investigation in animal models of IBD should be performed to assess their therapeutic use and as adjunct treatment with conventional therapeutics in IBD.

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CHAPTER 3

Flaxseed bioactives mitigate loss of intestinal epithelial barrier integrity in an *in vitro* co-culture model of inflammation

Ahmed A. Almousa, Sharyle Fowler, Jennifer Jones, Brian Bandy, François Meurens, Ed S. Krol, Jane Alcorn

Flaxseed bioactives mitigate loss of intestinal epithelial barrier integrity in an *in vitro* co-culture model of inflammation

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Brian Bandy[1], Ed S. Krol[1], Jane Alcorn[1*]

Transitioning rationale:

The previous chapter provided a proof of concept of the anti-inflammatory effects of enterolactone and linoorbitides on intestinal epithelial cells and an ability to maintain the intestinal epithelial barrier. However, to better simulate the pathophysiological conditions *in vivo*, in this chapter we incorporated a co-culture model that utilized the human Caco-2 (intestinal epithelium cell line) and mouse RAW 264.7 (macrophage cell line). RAW 264.7 cell lines can produce persistent inflammation upon induction with LPS, and thus, preserve features of IBD. This manuscript provides further evidence of LOBs and ENL positive effects on barrier integrity in the presence of stimulated macrophages (RAW 264.7). Moreover, the integration of the RAW 264.7 cell line provided a plausible element for the assessment of oxidative stress in this system. In addition, this manuscript continued the discovery process of potential inflammatory mediators involved in the observed effects of LOBs and ENL. Consequently, in this chapter the co-culture system enhances the relevance of the protective effects of LOBs and ENL on intestinal epithelial integrity and the possible inflammatory mediators.

Contribution statement:

Ahmed Almousa contributed to this manuscript by designing the study, performing experiments, data acquisition, data analysis and manuscript drafting.

Abstract

Flaxseed extract is rich with several bioactive components believed to possess beneficial effects in patients with inflammation related chronic disorders. However, no clear characterization exists of the role individual bioactives play in reducing inflammation and disease progression. Inflammatory bowel disease (IBD) is an important condition where the IBD population receives variable value from current treatments. New or adjuvant therapies are necessary to improve treatment outcomes and quality of life. Few studies have specifically evaluated the role of flaxseed bioactives in IBD. Hence, we assessed two major classes of flaxseed bioactives, linooribitides (LOBs) and the mammalian lignan, enterolactone (ENL), for their effect on the hallmarks of IBD pathogenesis, intestinal barrier integrity and oxidative stress. To this end, a co-culture of intestinal epithelial cells, Caco-2, and a macrophage cell line, RAW 264.7, were plated in a transwell system and lipopolysaccharide (LPS) was added to the basal compartment for 48 hours to stimulate an inflammatory response. Additionally, RAW 264.7 cells were used to assess protective effects upon exposure to reactive oxygen species. Results showed that ENL (2 μM) and LOB-ACEJ (20 nM) resulted in 1 and 1.5-fold increases in intestinal barrier resistance, respectively, when compared to LPS alone. Furthermore, ENL and LOBs were capable of reducing oxidative stress. Finally, upregulation of IL-5 was a signature for ENL in innate and adaptive immune response microarrays. Our results demonstrate that after an inflammatory insult ENL and the tested LOBs protect intestinal barrier integrity, reduce oxidative damage, and stimulate the immune system.

3.1 Introduction

Flaxseed is composed of three major components; omega-3 and alpha-linolenic acid (ALA), soluble and insoluble dietary fibers, and lignans. A fourth significant component of flaxseed, the cyclic peptides or linoorbitides (LOBs), have gained recent interest as potential bioactive compounds that add a significant contribution to the health benefits of flax [10, 26]. Although many human clinical trial interventions involving flax principally focused on the benefits of fibre and alpha-linolenic acid, the mammalian lignans and LOBs have gained more recent attention due to their potential to mitigate inflammatory conditions.

Inflammatory bowel disease (IBD) is an important condition where the IBD population receives variable value from current treatments. New or adjuvant therapies will be necessary to improve treatment outcomes and quality of life. The current shift in public attention to natural products as a safe source of medication with ease of access is being complemented by the current research which identifies the potential of these multicomponent plant extracts to target several pathways of pathological activity[271] . IBD has a complex pathogenesis and several *in vitro* and *in vivo* trials have utilized natural products with multi-target activity to ameliorate inflammation and help induce remission[272]. Few studies, though, have specifically evaluated the role of flaxseed bioactives in IBD.

The flaxseed lignan enterolactone (ENL), is a mammalian lignan that is detected in the systemic circulation upon ingestion of flaxseed standardized extracts[217] and is associated with reduced inflammatory markers like CRP in postmenopausal women and amelioration of inflammation after lung injury in mouse models [8, 9]. In addition, ENL has well studied antioxidant activity[52, 261]. ENL undergoes enterohepatic recirculation, which suggests increased concentration at the level of the gastrointestinal tract epithelium. This increased concentration is critical to elicit pharmacological activity in pathologies that are confined to the gastrointestinal tract like IBD. Conversely, LOBs have not been associated with an anti-inflammatory effect, but were more significantly attributed to immunosuppressive activity that is closely related to cyclosporin-A[26, 46, 47]. Due to their inherent large molecular weight (~1000 g/mol), LOBs are potentially incapable of crossing the intestinal epithelium[244] and, hence, likely to exert local effects on the epithelium.

Few studies have evaluated a role for the flaxseed bioactives, LOBs and lignans, in IBD, particularly studies which examine the activity of individual bioactives in IBD pathogenesis. Preliminary investigations typically employ *in vitro* model systems to assess the ability of plant

bioactive compounds in modulating the key components influencing the pathogenesis of IBD, namely the mucosal immune system, mucosal epithelial barrier function, and mucosal inflammation and oxidative stress[119, 120, 137, 273-275]. In the present study, we investigated the effect of flaxseed lignans and LOBs on intestinal epithelial barrier integrity, oxidative stress, and immune system by employing a co-culture of intestinal epithelial cells, Caco-2, and a macrophage cell line, RAW 254.7, in a transwell system[276]. This system results in a polarized epithelium with high integrity[277], that allows for relevant cell-cell communication between the two cell types[278], and inflammatory and oxidative stress responses that simulate conditions in the intestine [279,125, 274].

3.2 Materials and Methods

3.2.1 Reagents

Superoxide dismutase (SOD) and catalase (CAT) assay kits were purchased from Cayman Chemical (Ann Arbor, MI, USA). The SOD kit measures total SOD activity (Mn-SOD and Cu/Zn-SOD) and total of both cytoplasmic and mitochondrial SOD. The lipid peroxidation (MDA) assay kit was purchased from Abcam (Toronto, ON, Canada). RNA extraction was performed using Trizol and a Qiagen RNeasy mini kit (Toronto, ON, Canada). Innate & Adaptive Immune Responses PCR Array was purchased from QIAGEN (Toronto, ON, Canada). 2′,7′-Dichlorofluorescin diacetate (DCFH-DA), lipopolysaccharide (LPS) from Escherichia coli 0111:B4, enterolactone (ENL), fetal bovine serum (FBS) and 0.025% trypsin–EDTA were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Colorectal adenocarcinoma-C2BBe1 (Caco-2-BBe) and mouse macrophage (RAW 264.7) cell lines were acquired from American Type Culture Collection (ATCC) (Manassas, VA, USA). Corning™ Transwell (0.4 μM, 24-well plates; Fischer Scientific, Pittsburgh, PA, USA) was purchased from Fisher Scientific (Pittsburgh, PA, USA). Dulbecco's modified Eagle medium (DMEM)-high glucose and penicillin-streptomycin (10,000 U/mL) were purchased from Thermo-Scientific (Waltham, MA, USA). Protein concentrations were determined using the Pierce™ BCA Protein Assay Kit (Rockford, IL, USA). All other reagents were cell culture grade.

3.2.2 Caco-2-BB2 and RAW 264.7 Culture Conditions

Caco-2-BB2 and RAW 264.7 cell lines were cultured in DMEM-high glucose (0.3 g/L glutamine and sodium pyruvate) supplemented with 10% FBS and 1% penicillin/streptomycin, and maintained at 37°C with 5% CO_2 in a humidified atmosphere. Cells were kept in the

logarithmic growth phase by routine passage every 2 – 3 days using 0.025% trypsin–EDTA treatment for Caco-2 and physical scraping for RAW 264.7. All experiments were carried out using passage numbers for 11-19 for RAW 264.7 and 52-63 for Caco-2.

3.2.3 Co-culture of Caco-2-BB2 and RAW 264.7

Caco-2 and RAW 264.7 cells in DMEM supplemented media (10% FBS) were plated on Corning Transwells. Caco-2 was plated on the apical side at an average density of 5×10^4 cells per insert and allowed to polarize for 16 days. RAW 264.7 cells were plated on the basal side of the transwell at an average density of 1×10^4 cells per transwell on day 12 of Caco-2 polarization interval with adaptation of the reported method by Tanoue et al[276]. Media was replaced every other day (200 µL on the apical side and 600 µL on the basal end) using vacuum suction. Transepithelial electrical resistance (TEER) values were measured using Millicell ERS™ voltohmeter by EMD Millipore®, after 30 minutes of acclimatisation in a biosafety cabinet. To optimise the concentrations of LPS used, we assessed TEER values by comparing several LPS concentrations against vehicle control of 1%DMSO at 24 and 48 hours. On the experiment day, FBS supplemented media was removed and substituted with Hank's Balanced Salt Solution (HBSS) (no Calcium) combined with LPS (10 µg/mL) addition to the basal compartment of transwells, while the apical compartment received HBSS only. Four different treatments of LOB-A, LOB-J, LOB-ACEJ, and ENL were added simultaneously with LPS in three replicates at three different concentrations on three different experimental days. The concentration used did not induce any cytotoxic effect as per our previous assessment (data not shown). Each flaxseed bioactive was initially dissolved in 100% DMSO, and then serially diluted to 1% DMSO with FBS free media and used as a final working solution. TEER measurement was done at the beginning of treatment and after 4, 8, 12, 24 and 48 hours. Relative TEER values were calculated by dividing TEER values for each treatment at each experimental time point by TEER values of its corresponding LPS only well at the same time point.

3.2.4 Reactive Oxygen Species (ROS) Activity with DCFH-DA in RAW 264.7

RAW 264.7 cells were seeded onto a 96-well plate (10,000 cells/well), left to attach for 24 hours, and then treated with LOB-A, LOB-ACEJ, and ENL for another 24 hours. At the end of the 24 hour treatment, media was changed and replaced with media that contained 100 µM hydrogen peroxide (H_2O_2) and fresh flaxseed bioactives for another one hour. Finally, 25 µM DCFH-DA

was used for ROS detection. Readings were taken at 485 nM excitation and 525 nM emission wavelengths using a Biotek Synergy HT microplate reader (Fisher Scientific, Canada). For imaging purposes, a similar procedure was performed, but the concentration of H_2O_2 was reduced to 50 µM. For visualization, we used a ZOE fluorescence microscope (Bio-rad, Hercules, CA, USA)

3.2.5 Measurement of Superoxide Dismutase (SOD) Activity, Catalase (CAT) Activity and Malondialdehyde (MDA) Concentration

Upon optimization, 200 ng/mL LPS for 24 hours resulted in a sustained quantifiable increased enzymatic activity of SOD and CAT. SOD and CAT activity assays in the RAW 264.7 cell line were carried out by incubating cells with 200 ng/mL LPS and flaxseed bioactives, LPS alone, and 1% DMSO (vehicle control). After 24 hours of co-incubation, protein concentration was quantified using Pierce™ BCA protein assay kit and normalized and activity was assessed per the manufacturer protocol. For MDA production (end-product of lipid peroxidation), cells were co-incubated with 400 ng/mL LPS for 6 hours and quantified for protein concentration, then MDA quantity was evaluated per the manufacturer`s instruction. The high concentration of LPS (400 ng/mL) and short duration was capable of capturing lipid peroxidation, unlike the use of 200 ng/mL which did not.

3.2.6 Innate and Adaptive Immune Response Array

RAW 264.7 cells were seeded in 6-well tissue culture plates and treated with LPS 10 µg/mL and flaxseed bioactives pooled at (200 nM) for 24 hours, and then total RNA was extracted from cells cultured on transwells by using Trizol and a Qiagen RNeasy mini kit. RNA purity and quantity was determined spectrophotometrically (Nanoview spectrophotometer; GE Healthcare, Baie d'Urfe, Quebec, Canada) by measurement at 260 nm and the OD_{260}/OD_{280} ratio, respectively. Total RNA was then reverse transcribed using a Life Technologies™ SuperScript® VILO™ cDNA Synthesis Kit and used for array analysis in the RT^2 Profiler PCR array for mouse innate and adaptive immune responses per the manufacturer's instructions. The array analysis was performed using Applied Biosystems® 7300 system (Foster City, CA, USA). Data analysis was performed on GeneGlobe Data Analysis Center website as per manufacturer recommendations. Microarray results were verified using quantitative RT-PCR (qPCR). Briefly, RNA from transwells as indicated above was quantified using Power Sybr® mastermix. The polymerase chain reaction was

carried out using a one-step method where an initial incubation with reverse transcriptase took place at 42ºC for 30 minutes, followed by denaturation at 95ºC for 3 minutes, 40 cycles of annealing and extension for 15 seconds and 30 seconds, respectively, and eventually a dissociation curve per manufacturer settings. Specific primers for target genes were designed using Integrated DNA Technologies (https://www.idtdna.com/Primerquest/Home/Index). Primers sequences for target genes were: 5`- GCTTCCTGTCCCTACTCATAAA-3` (forward) and 5`-CCCACGGACAGTTTGATTCT-3` (reverse) for Il-5, 5`- GGAGAAACCTGCCAAGTATGA-3` (forward) and 5`-TCCTCAGTGTAGCCCAAGA-3` (reverse) for GADPH, which served as a housekeeping gene to normalize C_T values. The $\Delta\Delta C_T$ method was used to determine the fold change in mRNA expression levels.

3.2.7 Statistical Analysis

All data represent means ± SD. Effects of treatments relative to controls were evaluated by using one-way ANOVA followed by Dunnett's post-hoc test. Differences were considered significant at *p-value* < 0.05. All data analysis was performed using GraphPad Prism 5 software (San Diego, CA, USA).

3.3 Results

3.3.1 Flaxseed bioactives mitigate reductions in transepithelial electrical resistance (TEER) following a lipopolysaccharide (LPS)-induced inflammatory response

We conducted several optimization steps to first elicit an appropriate inflammatory response to allow assessment of the ability of the flaxseed bioactives to protect epithelial barrier integrity. The co-culture system was optimized for cell density and LPS concentration that could reduce TEER values and induce an inflammation that is moderate and potentially reversible. Figure 3.1 shows three different concentrations of LPS (10, 25 and 75 µg/mL) and their relative TEER values compared to 1% DMSO (control) at two time points (24 and 48 hours). Reduction in TEER values behaved in a concentration dependent fashion. A concentration of 10 µg/mL was used to carry out the rest of the experiments in transwells as it showed a mild reduction in relative TEER to 92.25 % at 24 h and a persistent reduction at 48 hours (relative TEER = 55.57%).

LOB-A relative TEER values showed a steady increase at only one concentration (2 µM), which plateaued between 8-24 hours and declined thereafter (Figure 3.2). In contrast, LOB-J at a concentration of 20 nM maintained a high relative TEER (~200% of LPS only wells) for a period of 48 hours. LOB-ACEJ, in a similar pattern to LOB-A, showed maintenance of high TEER for

24 h but TEER values declined with longer exposure periods. Increasing concentrations of ENL resulted in maintenance of higher relative TEER (Figure 3.2).

3.3.2 Flaxseed bioactives reduce oxidative stress response in RAW 264.7 macrophage cell line

To determine the ability of the flaxseed bioactives to mitigate oxidative stress, H_2O_2 was used to induce an oxidative stress in the murine macrophage cell line, RAW 264.7, and markers of oxidative stress, ROS levels, lipid peroxidation, SOD activity, and CAT activity were assessed. In the absence of flaxseed bioactives, H_2O_2 increased ROS levels 46 % in comparison to 1% DMSO control (Figure 3.3). 100 µM of H_2O_2 was used after optimizing several variables like RAW 264.7 cell count, sensitivity to H_2O_2 and the quantifiably of fluorescence. The amount of ROS produced by H_2O_2 treated cells, as observed using the DCFH-DA probe, was decreased when treated with LOB-A, -ACEJ and ENL. The highest percentage of reduction was observed at 2 µM and was 33%, 32.7%, 26.6% and 18.2% for LOB-ACEJ, ENL, LOB-A and LOB-J, respectively. In contrast, the amount of ROS produced by the LOB-J and LOB-A treated cells was not significantly reduced at the lowest concentration of 20 nM (Figure 3.3). When challenged with lower concentrations of H_2O_2, and examined under the microscope, all flaxseed bioactives at 200 nM showed reduced fluorescence intensity when compared to H_2O_2 alone as seen in Figure 3.4.

Following LPS stimulation, assessment of SOD enzymatic activity in RAW 264.7 cells showed that ENL (0.2 µM and 20 µM) significantly increased SOD activity by 35% and 62% relative to LPS only treatment (Figure 3.5). Among the cyclic peptides, only LOB-A at a high concentration (2 µM) was capable of increasing (79.5%) SOD activity following LPS induced ROS production (Figure 3.5). Catalase activity was increased with ENL and LOB-J (20 µM) by 44.3% and 42.6% respectively (Figure 3.6), relative to LPS only treatment. To assess lipid peroxidation 400 ng/mL of LPS was co-incubated with the flaxseed bioactives for 6 hours, and each bioactive caused a concentration-dependent reduction in lipid peroxidation with high concentrations of LOBs reducing MDA production (Figure 3.7) by 43.5%, 40.8% and 32.5% for LOB-J, LOB-A and LOB-ACEJ (all at 2 µM), respectively. ENL at 20 µM significantly reduced MDA production by 36.9% in comparison to LPS only treatment.

3.3.3 Upregulation of IL-5 as a signature of flaxseed bioactives

Innate and adaptive immune response gene expression revealed six genes that were significantly altered (Figure 3.8). Those upregulated genes were signal transducer and activator of

transcription 4 (Stat4) and interleukin-5 (IL-5). Down-regulated genes were chemokine receptor 6 (Ccr6), CD40 ligand (Cd40Ig), Fas ligand (Fasl) and interleukin-13 (IL-13).

To confirm the results obtained from the microarray, we performed independent qPCR with a different primer design tool. When independently verified, only IL-5 provided reproducible results that could be replicated in a different experiment with homemade primers. When broken down to single bio-actives treatment, ENL was mainly responsible for the upregulation of IL-5 in dose response trend as in figure 3.9. Verification of microarray results using qPCR showed a significant 2.85 and 5.3-fold increase in IL-5 expression relative to 10 µg/mL LPS control at 2 and 20 µM respectively. On the other hand, LOBs did not change the expression of IL-5 at the same concentration as ENL.

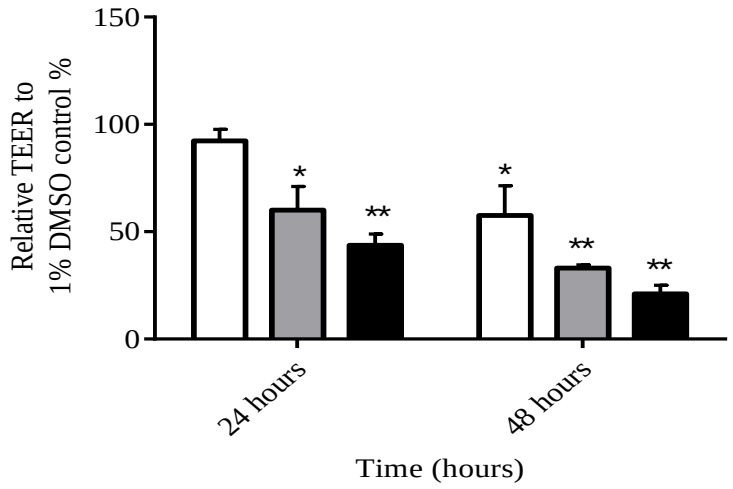
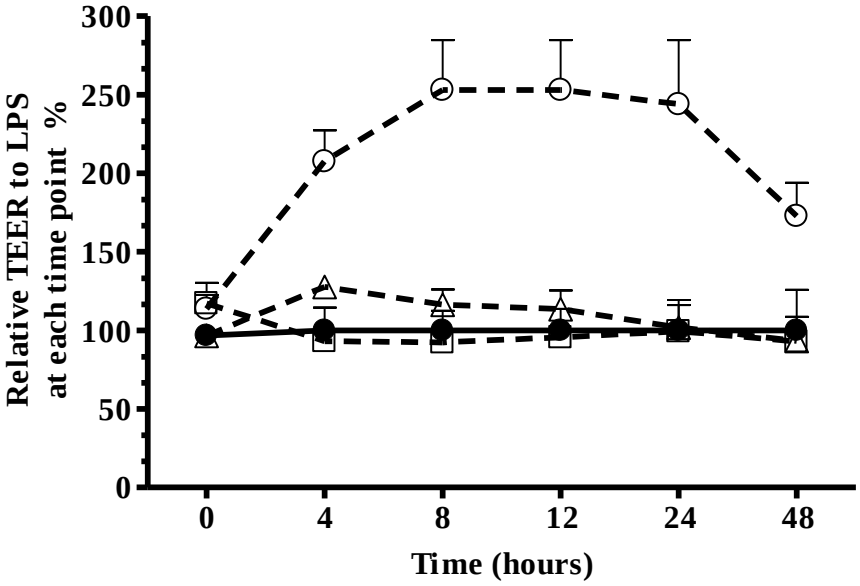
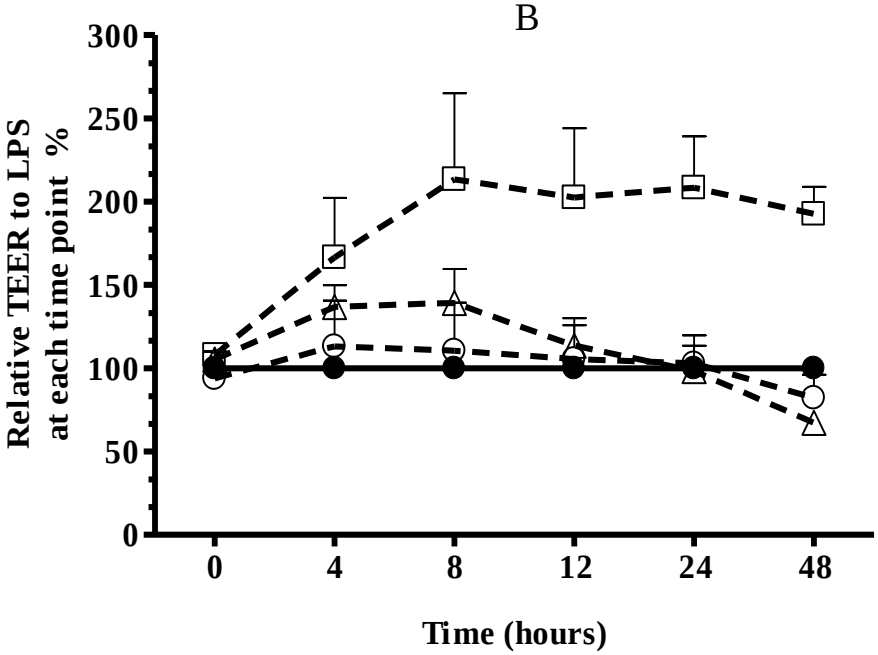


Figure 3.1 Effect of different lipopolysaccharide (LPS from E-coli 0111:B4) concentrations (open bar, 10 µg/mL; grey bar, 25 µg/mL; black bar, 50 µg/mL) on transepithelial electrical resistance (TEER) values relative to vehicle control (1% DMSO) in a co-culture system of RAW 264.7 and Caco-2 cells at 24 and 48 hours. * p-value < 0.01and **p-value < 0.001.

A


Relative TEER to LPS
at each time point %
300
250
200
150
100
50
0
0
4
8
12
24
48
Time (hours)

B


Relative TEER to LPS
at each time point %
300
250
200
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100
50
0
0
4
8
12
24
48
Time (hours)

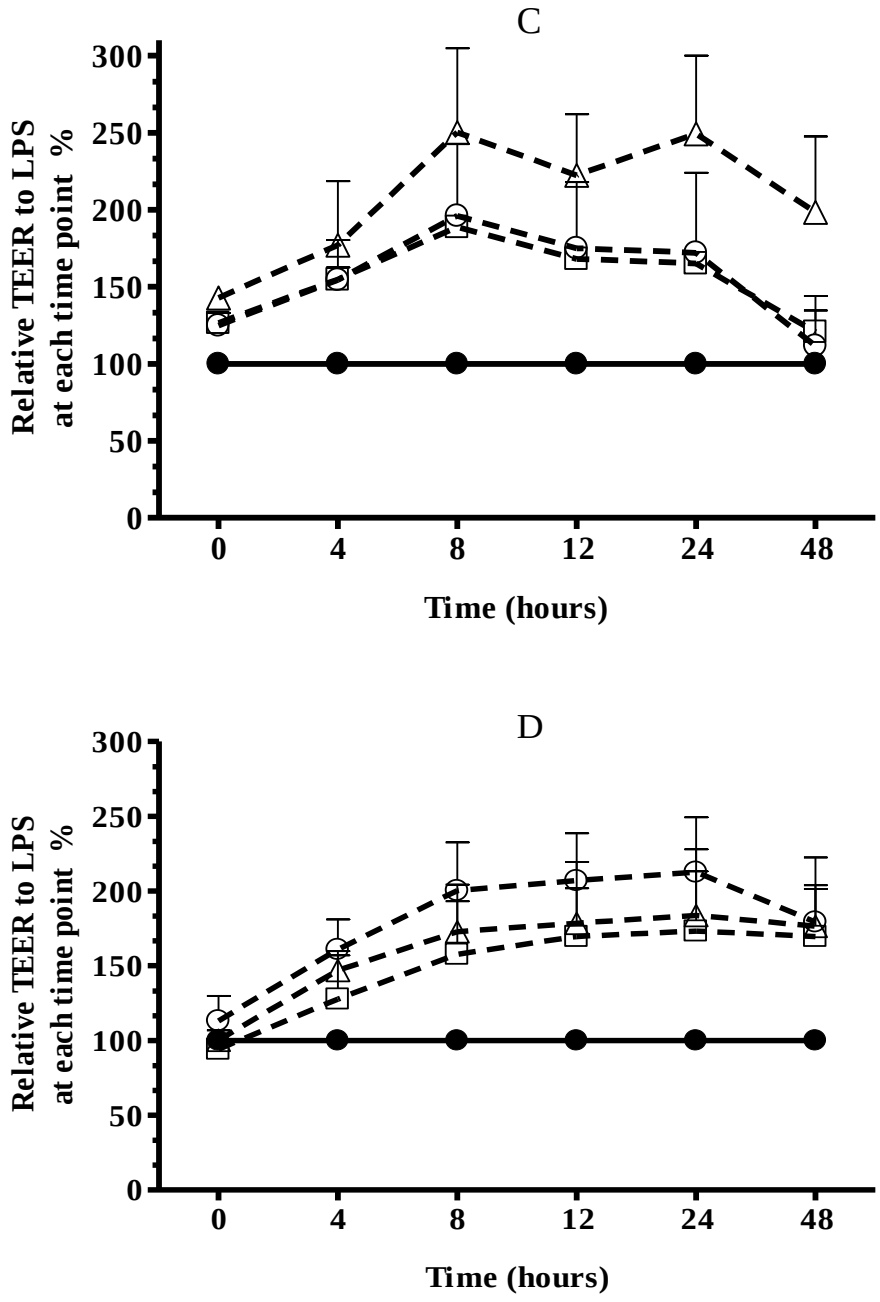

Figure 3.2 Relative TEER values of flaxseed bioactives to LPS (from E-coli 0111:B4) only treated wells at each corresponding time point. A, B, C and D represents the simultaneous treatment of LOB-A, LOB-J, LOB-ACEJ and ENL with LPS exposure, respectively. Open circles (○) represent 2 µM, open triangles (Δ) represent 200 nM, open squares (□) represent 20 nM, and closed circles (●) represent LPS 10 µg/mL. Data are represented by means ± SD with four replicates at each occasion in three different experimental days.

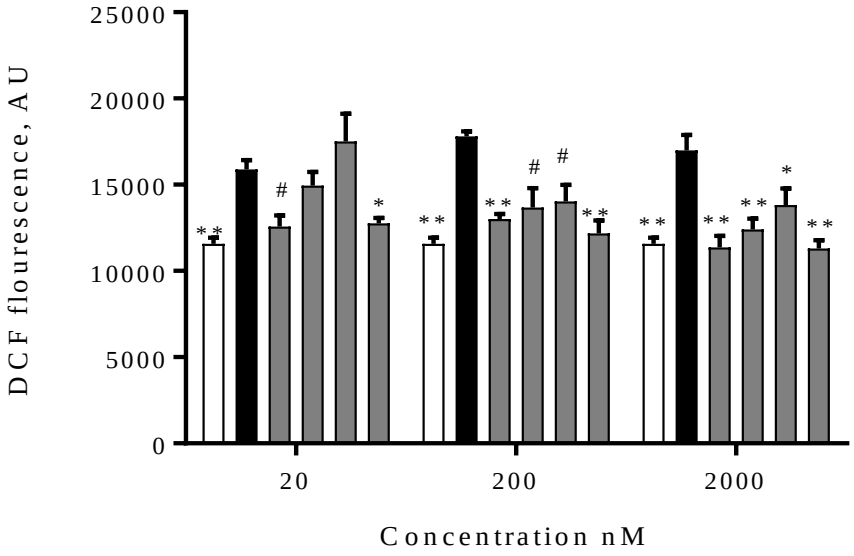

Figure 3.3 Effect of different bioactives on DCFH-DA oxidation in RAW 264.7 cells upon induction with 100 µM H_2O_2 for 24 hours. (open bar, 1% DMSO; black bar, 100 µM H_2O_2; dark gray bars from left to right, ENL, LOB-A, LOB-J and LOB-ACEJ). Three different concentrations of bioactives were used (20, 200 and 2000 nM). Data are presented as means ± S.D, n=3. * p-value < 0.05, #p-value < 0.01 and **p-value < 0.001.

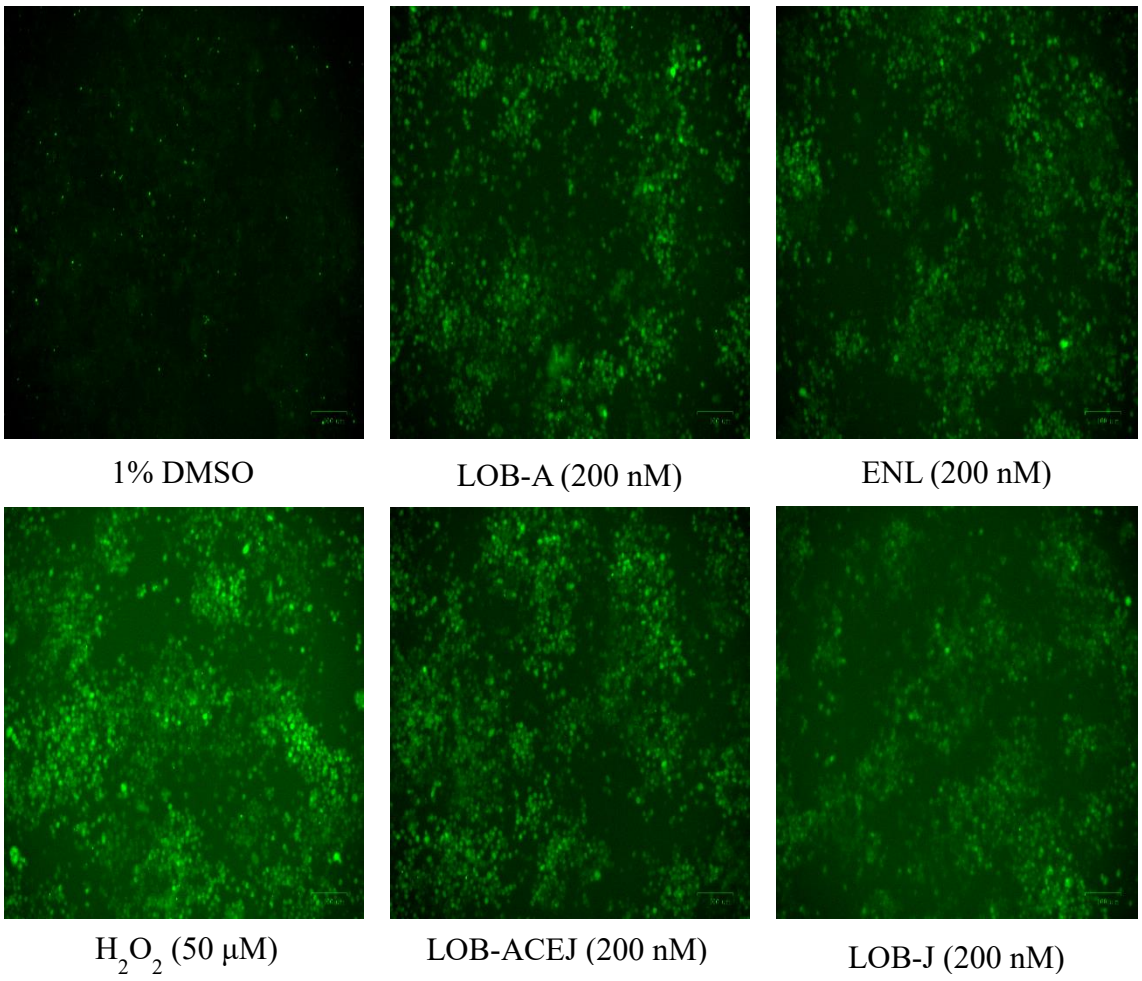

Figure 3.4 Effect of 200 nM of linoorbitoids (LOBs) and enterolactone (ENL) on the intracellular oxidation of the reactive oxygen species (ROS) probe DCFH-DA. Images were taken with a ZOE fluorescence microscope.

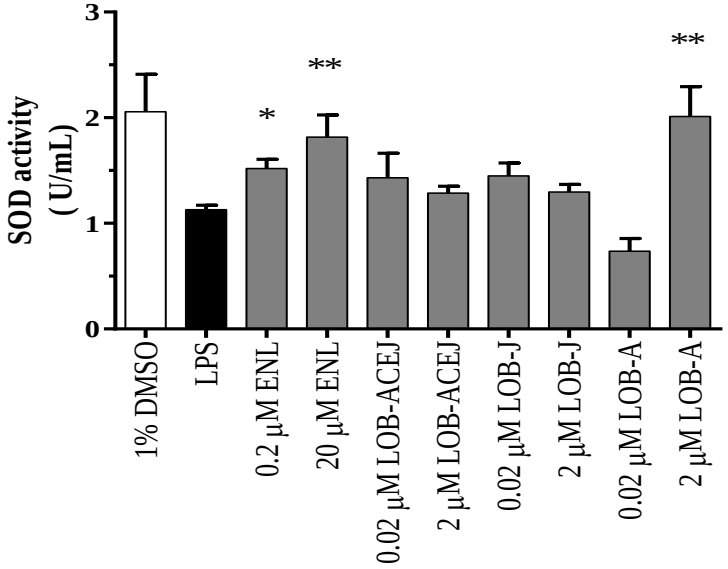

Figure 3.5 Effect of LOB-A, -J, -ACEJ and ENL on SOD activity in RAW 264.7 cells upon co-treatment of flax bio-actives with LPS (200 ng/mL from E-coli 0111:B4) for 24 hours. Data are presented as means ± S.D, n=3. One way ANOVA was performed and data were compared to LPS alone, * p-value < 0.05, ** p-value < 0.01.

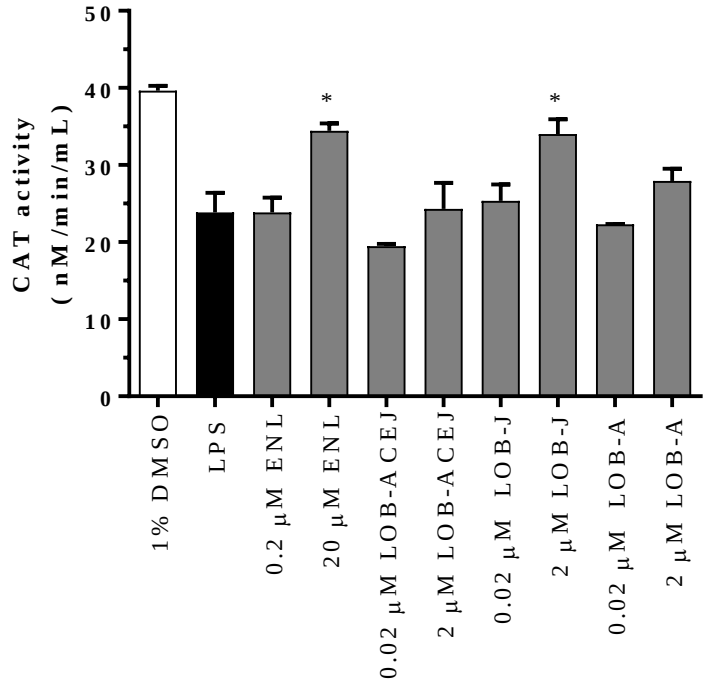

Figure 3.6 Effect of LOB-A, -J, -ACEJ and ENL on CAT activity in RAW 264.7cells upon co-treatment of flax bio-actives and LPS (200 ng/mL from E-coli 0111:B4) for 24 hours. Data are presented as means ± S.D, n=3. One way ANOVA was performed and data was compared to LPS alone, * p-value < 0.05.

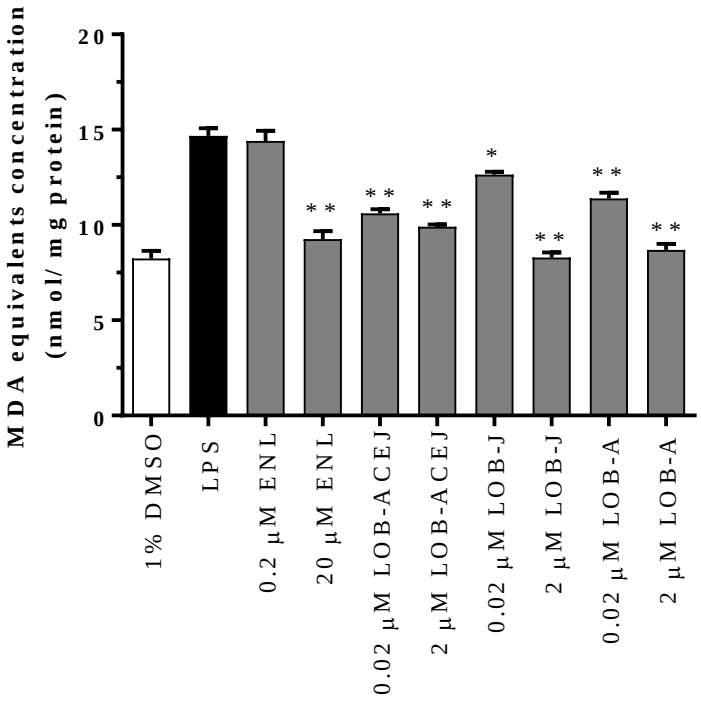

Figure 3.7 Effect of LOB-A, -J, -ACEJ and ENL on MDA concentrations in RAW 264.7cells upon co-treatment of flax bio-actives and LPS (400 ng/mL from E-coli 0111:B4) for 6 hours. Data are presented as means ± S.D, n=3. One way ANOVA was performed and data were compared to LPS alone, * p-value < 0.05, ** p-value < 0.01.

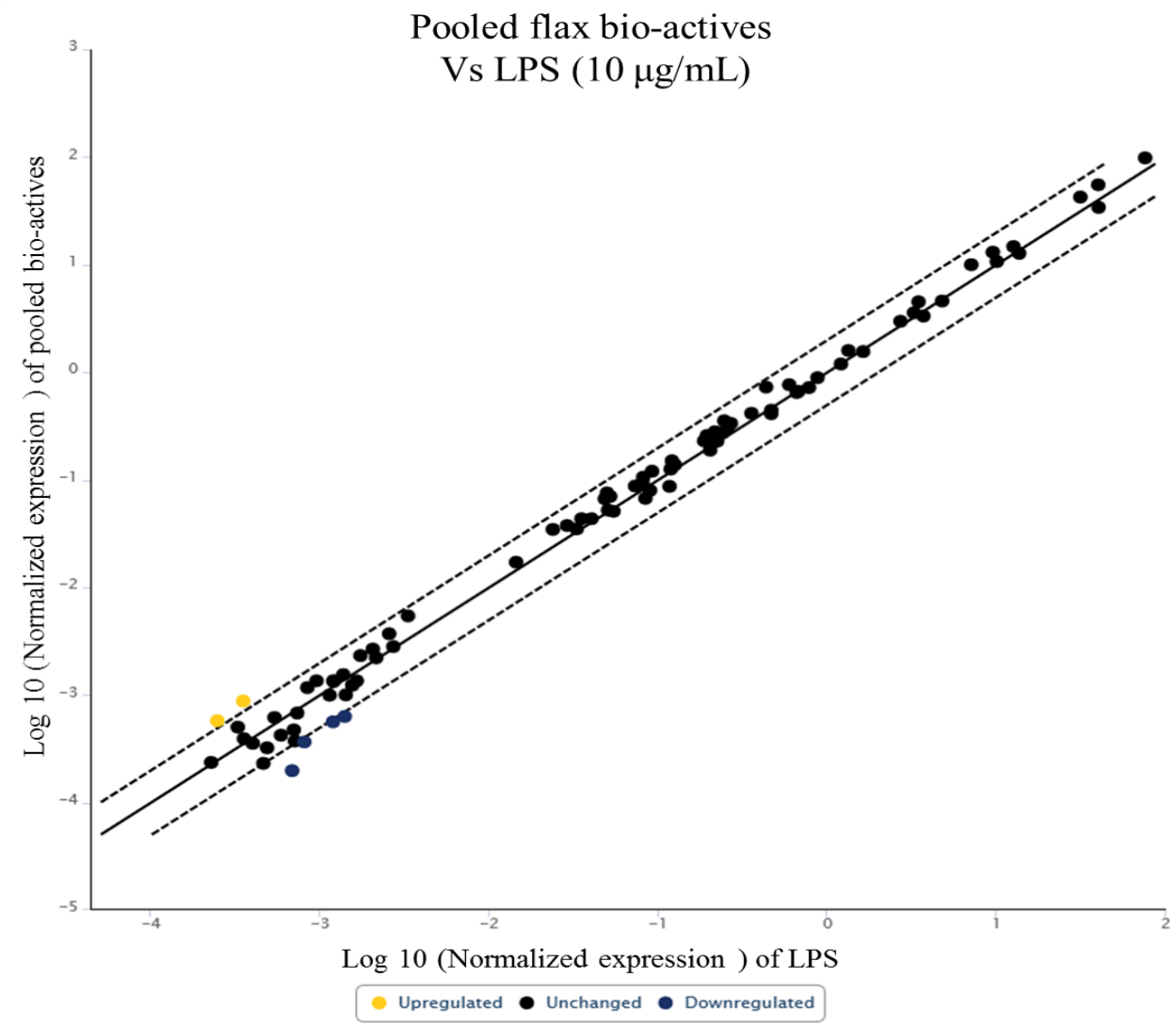

Figure 3.8 Innate and adaptive immune response microarray in RAW 264.7 cells after co-treatment of pooled flaxseed bioactives (equimolar ratios of ENL, LOB-A, LOB-J and LOB-ACEJ at 200 nM each) and 10 µg/mL LPS from E-coli 0111:B4. Solid black line (—) represent zero-fold change in expression between pooled treatment and LPS alone. Dashed line (----) represents the two folds' regulation that used as a cut-off for significant difference between control and treatments groups. Upregulated genes (Yellow circles (●)) were Stat4 and IL-5, down-regulated genes (Dark blue circles (●)) are Ccr6, Cd40Ig, Fasl and Il-13 at p-values of < 0.05.

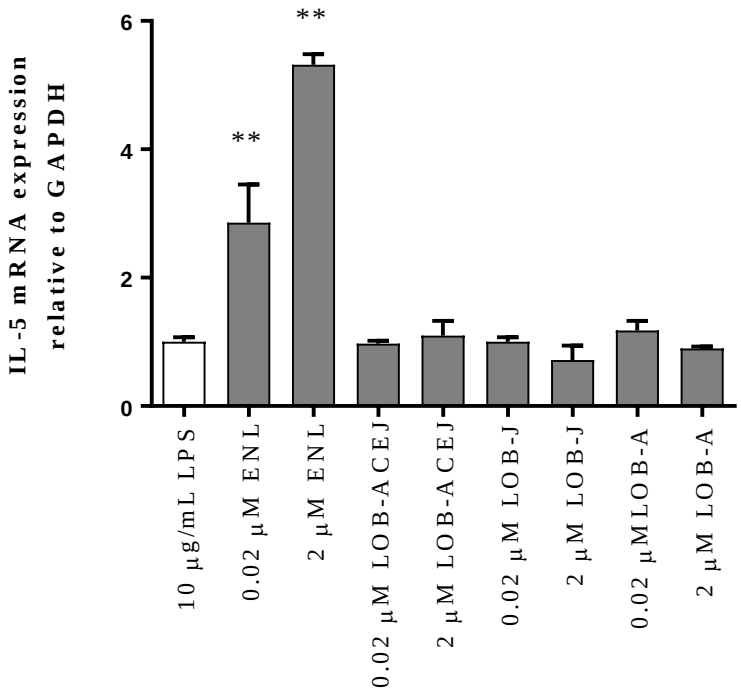

Figure 3.9 Microarray verification with independent assay confirmed the upregulation of IL-5 by ENL. Verification was carried out for LOB-A, -J, -ACEJ and ENL on IL-5 in RAW 264.7cells upon co-treatment of flax bio-actives and LPS. Data are presented as means ± S.D, n=3. One-way ANOVA was performed and data were compared to LPS alone, ** p-value < 0.001.

3.4 Discussion

Flaxseed lignans and the cyclic peptides LOBs are purported to have anti-inflammatory, antioxidant and immune modulatory effect[26, 44, 261]. Lignans and LOBs have gained attention in other chronic diseases but have limited investigation in IBD. Therefore, this study investigated the lignan, ENL, and several LOBs (LOB-A, -J and -ACEJ) for their ability to maintain barrier integrity, reduce oxidative stress, and as potential immune modulators, as key hallmarks of IBD pathogenesis. This study used an *in vitro* co-culture model of Caco-2 (intestinal epithelium) and RAW 264.7 (macrophages) cell lines, to acknowledge the importance of intestinal epithelial cell and macrophage interactions in the pathogenesis of IBD[276, 280, 281]. Further, lipopolysaccharide stimulation of toll-like receptor-4 of macrophages triggers an inflammatory cascade in the co-culture with induction of oxidative stress that simulates events in the inflamed epithelium of IBD patients[146]. This behaviour was necessary to allow evaluation of the anti-inflammatory effects of ENL and LOBs and their effects on oxidative stress.

Currently it is believed that losing integrity of intestinal epithelial barrier function is a critical part of IBD pathophysiology[119, 120, 137, 275]. In this study, barrier integrity protection in the co-culture model was generally maintained with ENL and the LOBs, although a reverse concentration-response relationship was noted with LOB-A. These results are consistent with effects noted in a monoculture of HCT-8 polarized epithelium (Chapter 2) although concentrations up to 2 µM were necessary in this study to ensure that the intended effects were observed given enhanced inflammatory status mediated by LPS stimulation of macrophages. With regard to the LOBs, the suppression of the immune response initiated from macrophages (RAW 264.7) is supported by the reported immunosuppressive effects of LOBs in general[47, 48]. The concentration-dependent differences noted with the LOBs is likely due to the potency of immunosuppressive activity of certain LOBs. Immunosuppressants such as methotrexate are known to modulate inflammation in pathologies like rheumatoid arthritis[282], while at higher concentrations and increased dosing frequencies it is utilized as an anticancer agent through its ability to induce cancer cell apoptosis[283]. In this study, LOB-A had the greatest potency with respect to immunosuppressive activity, and at higher concentrations may induce cell apoptosis. Therefore, LOB-A at higher concentrations might cause an apoptotic effect on RAW 264.7 cells (the source of inflammatory mediators) and reduce the intensity of the inflammatory insult or epithelial cell apoptosis and reduction in barrier integrity.

The ability of ENL to decrease oxidative stress in the macrophage cell line is consistent with the reported anti-oxidant activity of flaxseed lignans in general, an activity that is attributed to their diphenolic chemistry and capacity to scavenge free radicals through donation of an electron, more specifically from the 4-hydroxy substituents of SDG and SECO, and the meta mono-phenol structures of END and ENL[52]. The ability of ENL to increase expression of the antioxidant enzymes, CAT and SOD, contributes to the reduction in oxidative stress, which is also consistent with reported ability of the lignans to upregulate hepatic CAT and SOD activity and expression, and glutathione peroxidase expression *in vivo* under different pathological contexts (diabetes, cardiac damage, and liver fibrosis)[284, 285]. Interestingly, the LOBs generally had a more pronounced reduction in lipid peroxidation without consistent upregulation of CAT and SOD activity. The high molecular weight of LOBs and their potential lack of cellular uptake likely confines their effect to reductions in extracellular reactive oxygen species (ROS), which would explain the reduction in MDA peroxidation without changes in intracellular antioxidant enzyme

expression. The increased SOD and CAT activity at high concentrations of LOBs could be a result of cellular homeostasis in response to extracellular ROS status rather than directly influencing the activity of such enzymes. Furthermore, the beneficial effect of LOBs on lipid peroxidation might be due to the short duration of the inflammatory insult (6 hours vs 24 hours for the SOD and CAT experiment), which could have captured the high burst of ROS generation during the initial stages of LPS stimulation. However, these experiments were limited by the lack of understanding of LOB permeability, which is necessary to understand whether they exert intracellular or extracellular activity.

Although cytokine and chemokine-mediated recruitment is essential for maintenance of barrier integrity[149], enhanced expression of IL-5 in LPS stimulated macrophages with ENL treatment was unexpected. Elevation of inflammatory biomarkers such as IL-6, TNF-α and CRP, in IBD is typically reported in the literature[286, 287], but IL-5 is an eosinophil activating cytokine[288] believed principally to be responsible for clearing parasitic, viral and bacterial infection in the gastrointestinal tract[289-291]. This effect might suggest a potential role of ENL in prophylaxis of intestinal inflammation rather than treatment of active disease where activation of eosinophils might be more deleterious[288]. Interestingly, in a DSS colitis mouse model activation of the eosinophilic pathway was associated with attenuation of inflammation[292], and further this is indirectly supported by the incidence of eosinophilic activation as a result of 5-ASA treatment, a mainstay treatment in IBD [293, 294].

In conclusion, flaxseed LOBs and ENL provide significant protection of epithelial barrier integrity in a co-culture model of macrophage and intestinal epithelial cells. Protection of barrier integrity was supported by LOB and ENL mediated reduction in oxidative stress, an effect likely the result of different mechanisms of action between the LOBs and ENL. In addition, ENL and LOBs reduced ROS generated lipid peroxidation in macrophages and upregulated IL-5 to modulate the immune response. Further assessment of ENL and LOBs in a colitis animal model is warranted to confirm their *in vivo* effect on barrier integrity, oxidative damage, and immune system modulation to mitigate these hallmarks of IBD pathogenesis.

CHAPTER 4

Serum Pyruvate Kinase M2 (PKM2) is Elevated in Inflammatory Bowel Disease

Ahmed A. Almousa, Marc Morris, Sharyle Fowler, Jennifer Jones, Jane Alcorn

Serum Pyruvate Kinase M2 (PKM2) is Elevated in Inflammatory Bowel Disease

Ahmed A. Almousa[1], Marc Morris[1], Sharyle Fowler[2], Jennifer Jones[3], Jane Alcorn[1*]

Transitioning rationale:

The previous chapter explored the effects of flaxseed bioactives on an *in vitro* coculture model of intestinal inflammation. Through the previous chapter, we assessed the effect of pooled flaxseed bioactives on innate and adaptive immune response gene microarray. To further understand the mechanism involved we applied the microarray results into gene set enrichment software. Results from this screen identified L-phenylalanine as a potential metabolite strongly associated with this screen enrichment. L-phenylalanine is a potent allosteric inhibitor of pyruvate kinase M2 (PKM2), an enzyme considered to exist at the interphase between inflammation and cellular metabolism. To enhance the translational aspect of this thesis, this following chapter investigated the utility of PKM2 as a potentially useful biomarker in IBD.

Contribution statement:

Ahmed Almousa contributed to this manuscript by designing *in vitro* studies, performing *in vitro* experiments, data acquisition for PKM2 serum concentration, data analysis and manuscript drafting. Marc Morris designed and collected human clinical samples (feces, blood and serum), clinical data acquisition, analyzed clinical samples for disease activity and microbial content.

Abstract

In inflammatory bowel disease (IBD) endoscopy remains the gold standard to diagnose and evaluate disease activity. The available biomarkers or their combinations cannot adequately predict IBD disease risk, diagnosis, progression or relapse, and response to therapy. A need continues for identification of more predictive serum biomarkers. Pyruvate kinase M2 (PKM2) is suggested to be a significant mediator of the inflammatory process. To evaluate a role for PKM2 as a potential biomarker in IBD we assessed serum samples from newly diagnosed IBD patients for PKM2 levels. We measured serum PKM2 by ELISA and correlated levels with several disease activity scoring systems, IBD disease type, and intestinal microbiota in newly diagnosed IBD patients. Furthermore, we tested the genetic expression of PKM2 after exposure of a *in vitro* intestinal cell lines to lipopolysaccharide (LPS) in the absence and presence of novel anti-inflammatory molecules of flaxseed currently under investigation in our laboratory. When compared against standard healthy controls, serum PKM2 levels were 6-fold higher in IBD patients, with no sensitivity to disease phenotype (Crohn`s Disease or Ulcerative Colitis) or localization of inflammation, and had less interindividual variability than established IBD biomarkers. We also observed a positive correlation ($r = 0.6121$) between serum PKM2 and *Bacteroidetes* fecal levels in Crohn`s disease (CD), in addition to a negative ($r = -0.6128$) correlation between serum PKM2 and *Actinobacteria* fecal levels. Furthermore, LPS (500 ng/mL) significantly upregulated PKM2 expression *in vitro*, which was significantly suppressed by the flaxseed bioactive, Linoorbitide-A (LOB-A), at 200 nM. In conclusion, our data suggests PKM2 as a putative biomarker for IBD and the dysbiosis of microflora in IBD. Furthermore, we continue to develop a body of evidence for the potential utility of flaxseed bioactives in IBD.

4.1 Introduction

IBD presents in two different distinct pathologies, Ulcerative Colitis (UC) and Crohn`s Disease (CD)[137]. IBD is a very important chronic inflammatory disease with a significant burden on the health care system and the quality of life for those who suffer from it[186, 295]. Currently, several biomarkers and their combinations are considered to aid diagnosis of IBD including C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), fecal lactoferrin, fecal calprotectin and calgranulin C[188]. In addition, biomarkers to distinguish between IBD phenotypes have been utilized in clinical practice to help assess the severity of disease including anti-*Saccharomyces cerevisiae* antibody, anti-glycoprotein-2, anti-granulocyte macrophage colony-stimulating factor, anti-neutrophil cytoplasmic antibody, anti-mannobioside carbohydrate IgG antibody, and anti-CBir1[189]. However, these biomarkers lack sufficient sensitivity and specificity[190-192 193, 194] and endoscopy remains the gold standard to diagnose and to stage disease severity in IBD patients[189, 195]. The identification of alternative less invasive biological markers to adequately predict IBD disease risk, diagnosis, progression or relapse, and response to therapy remains an urgent need in the field of gastroenterology.

A recent abstract published by Marin *et al*. identified a significant correlation between PKM2 levels and active UC[296]. Furthermore, PKM2 is gaining attention as a mediator of the inflammatory process by acting as a bridge between energy metabolism and inflammatory cellular processes[297]. Elevated PKM2 has been associated with patients diagnosed with lung cancer, renal cancer, ovarian cancer, and melanoma[298-301]. Pyruvate kinase (PK) regulates the final step of glycolysis by catalyzing the transfer of a phosphate group from phosphoenolpyruvate (PEP) to ADP to produce ATP and pyruvate[302]. PK is involved in the Warburg effect in cancer cells, and its activity is a determinant of cell proliferation through the shunting of metabolites into the pentose phosphate pathway to support nucleotide biosynthesis required by a rapidly dividing cell, whether it is cancerous or epithelial [303-305]. On the cellular level, the exact role of PKM2 in the inflammatory process is not known, but proteomic profiling of neutrophilic granules showed high abundance of PKM2 as an evidence for critical role in immune cell recruitment[306]. In the intestinal epithelium, PKM2 upregulation is believed to impart a favourable effect by repressing apoptosis[305]. In addition, the high turnover of intestinal epithelial cells is considered a shared characteristic with cancerous cells.

This study aimed to confirm an association with serum PKM2 levels and patients newly diagnosed with IBD and whether it may differentiate between CD and UC. Moreover, this study assessed whether PKM2 elevation is observed in an *in vitro* model of intestinal inflammatory stimulation and whether novel bioactives from flaxseed may ameliorate elevations in this enzyme.

4.2 Materials and methods

4.2.1 IBD patient recruitment and assessment

Newly diagnosed (within the past 12 months) IBD patients as determined by the treating physician at the Royal University Hospital and Saskatoon area were considered eligible subjects. Individuals ages 18 and older with a diagnosis of IBD based on Lennard-Jones *et al.* criteria[307], and able to provide a written informed consent participated in the study. The study was conducted between November 2012 to July 2014[307]. Several disease activity scoring modules were utilized, including; Crohn`s Disease Activity Index (CDAI), IBDQ, Mayo in UC, and Harvey scoring for CD[308-310]. Exclusion criteria included patients with end-stage kidney disease, cardiovascular disease, infectious disease or hepatic disorders or who are pregnant. This study was approved by the University of Saskatchewan's Biomedical Research Ethics Board (Bio-REB) (Bio# 09-26).

At baseline and follow-up visits (3-6 months) over the two-year period, endoscopy, disease activity scores, fecal collection and blood work were performed for each participant in the trial. Fecal samples were aliquoted (50 gm) and frozen immediately and sent out for microbiota analysis by Contango Strategies Limited (Sasktoon, SK).

Fecal calprotectin was measured using a Quantum Blue® FC High Range Rapid Test using a company recommended reader (AlPCO, Salem, NH, USA). For this purpose, 30 mg of fecal sample was added to a base cap and subsequently fitted for extraction. Then, 4 mL of extraction buffer (provided with the kit) was added and the mixture was vortex-mixed for one minute. A sixteen times dilution (with chase buffer) was performed and only 80 µL was used in the reader. Fecal lactoferrin was determined using IBD-SCAN® (TechLab, Blacksburg, VA, USA). A 450 mg fecal sample was transferred to an Eppendorf tube and diluted at 1:100 to 1:10,000. Samples and controls were plated on a 96-well plate provided with the kit and read at 450 and 620 nm excitation and emission, respectively, using a Biotek Synergy HT microplate reader (Fisher Scientific, Canada).

4.2.2 PKM2 Enzyme Linked-Immunosorbent Assay (ELISA) in human IBD patients

Serum from thirty-three newly diagnosed IBD participants was collected and used to quantify their PKM2 content. In a 96-well plate, 100 µL of sample was diluted with 100 µL Dulbecco's phosphate-buffered saline (DPBS) buffer, then 100 µL was added to each well and the measurement was carried according to the manufacturer's protocol (Elabscience Ltd., Bethesda, MD). These samples were compared against standard control serum purchased from Biocell Laboratories (Rancho Dominguez, CA, USA). In addition, PKM2 correlation analysis (see statistical methods for details) was conducted against disease localization, disease activity score, fecal microbiome content and other standard IBD biomarkers (fecal calprotectin and fecal lactoferrin).

4.2.3 Cell culture

The colon adenocarcinoma epithelial cell line (Caco-2-BB2) and the mouse macrophage (RAW 264.7) cell line were acquired from American Type Culture Collection (ATCC) (Manassas, VA, USA) and maintained in DMEM-High Glucose media supplemented with 10% bovine serum albumin and 1% penicillin/streptomycin (10,000 U, 10 mg/mL) from Sigma-Aldrich (St. Louis, MA, USA). Initially, cells were passaged twice after removal from liquid nitrogen, media changed every two days, and cells in Corning™ T-75 flasks (Pittsburgh, PA, USA) were passaged at 70-80% confluency using 0.25% Trypsin-EDTA (Sigma-Aldrich, St. Louis, MA, USA). Cells were maintained at 37°C and 5% CO_2 all the time. For co-culture studies, a combination of both Caco-2 and RAW 267.7 cell lines were added in a ratio of 100:1 to 6-well plates Corning™ Costar (Pittsburgh, PA, USA). Cell cultures were used at passage numbers 13-20 for RAW 264.7 and 52-60 for Caco-2 for all experiments.

4.2.4 Optimisation of PKM2 regulation in Caco-2 and verification of inflammation reduction with LOB-A

After optimization, we used Caco-2 cells in DMEM-High Glucose supplemented media plated on 6-well plates at a seeding density of 1×10^5 cells per well. Cells were allowed to grow for a minimum of three days to achieve high confluency levels (~80%). At the time of inflammation induction, supplemented media was removed and substituted with serum free media with varying concentrations of lipopolysaccharide (LPS) from Escherichia coli 0111:B4 or TNF-α (Sigma-Aldrich, St. Louis, MO, USA) or both for either 24 or 48 hours. After induction of inflammation,

total RNA was extracted from cells by using Trizol and Qiagen RNeasy mini kit (Toronto, ON, Canada) following the manufacturer protocols. Total RNA was then reverse transcribed by Life 5X All-in-one RT Mastermix (Applied Biological Materials) to cDNA, and then gene expression quantified using Power Sybr® Green PCR Mastermix (Burlington, ON, Canada). The quantitative polymerase chain reaction was carried out on an Applied Biosystems® 7300 system (Foster City, CA, USA) with denaturation at 95ºC for 3 minutes, and after denaturation, 40 cycles of annealing at 95ºC and extension at 60ºC for 15 seconds and 30 seconds each, with a final dissociation curve per manufacturer setting. Primer sequences for target genes were: 5`-GCTGACTCCTGCATAGGTTATC-3` (forward) and 5`-GCGAATGCCTCAGAGTAGAAA-3`(reverse) for PKM2, 5`- CAAGAGCACAAGAGGAAGAGAG-3` (forward) and 5`-CTACAT GGCAACTGTGAGGAG-3` (reverse) for GAPDH, which served as a housekeeping gene to normalize Ct values. The Delta-Delta threshold cycle ($\Delta\Delta C_T$) method was utilized to determine fold change in mRNA expression levels.

Similar to the experimental design used for RNA expression, we quantified PKM2 protein expression after prolonged (48 hours) and high LPS activation (500 ng/mL). Cells were collected from 6-well plates using Trypsin-EDTA 0.25% then were centrifuged at 1000 *g* for 5 minutes and the pellet was reconstituted with one mL of DPBS. Subsequently, cells were frozen and thawed three times and homogenized with a microtube homogenizer for one minute on ice. The homogenate was centrifuged again at 1000 *g* for 20 minutes, and 100 µL of clear supernatant was added to the assay for assessment of PKM2 expression per manufacturer protocol (Elabscience Ltd., Bethesda, MD).

4.2.5 Statistical Analysis

Data are presented as mean ± SD unless otherwise mentioned. Effects of treatments relative to control were evaluated by using one-way ANOVA followed by Dunnett's post-hoc test. Differences were considered significant at *p-value* < 0.05. All data analyses were performed by using GraphPad Prism 5 software (San Diego, CA, USA). Correlation analysis was also performed with GraphPad Prism 5 using two tailed correlations with linear regression of 95% confidence intervals and an assumption of data being sampled from a Gaussian population.

4.3 Results

4.3.1 Serum PKM2 levels are elevated in newly diagnosed IBD patients

Serum samples from 33 IBD patients were quantified for PKM2 concentrations using ELISA and compared with serum from four standard controls. Mean serum PKM2 levels were approximately six times higher than control serum (Figure 4.1). Serum PKM2 was compared against well-established IBD biomarkers, fecal calprotectin and fecal lactoferrin, from the same patients and demonstrated less variability of quantiles (Figure 4.2). When participants were stratified according to IBD diagnosis (location of inflammatory lesions by endoscopic evaluation), levels of PKM2 were not statistically significant with endoscopic results (data not shown). Similarly, there was a significant increase in serum PKM2 in both UC and CD patients compared to control, with no difference between UC and CD (Figure 4.3). To determine whether PKM2 serum levels related to disease activity scores, we conducted a correlation analysis of PKM2 concentrations with different disease activity score categories. Figure 4.4 indicated no significant correlation between serum PKM2 and disease activity indices.

Among several types of intestinal microflora analyzed (*Actinobacteri*, *Bacteroidetes*, *Cyanobacteria*, *Firmicutes*, *Fusobacteria*, *Proteobacteria*, *TM7*, *Tenericutes* and *Verrucomicrobia*) for the 33 IBD patients only two types of bacteria showed significant correlation with PKM2 serum concentration. Both *Bacteroidetes* and *Actinobacteria* were significantly correlated to PKM2 concentrations in patients with CD, where increased PKM2 corresponded to an increase in *Bacteroidetes* ($r = 0.6121$) and reduction in *Actinobacteria* ($r = -0.6126$) (Figure 4.5).

4.3.2 PKM2 regulation is dependent on inflammation duration and cell line

Initially two different types of culture systems were tested, a mono-culture of Caco-2 and a co-culture of Caco-2 and RAW 264.7. The two systems were treated with different concentrations of inflammatory inducers, TNF-α or LPS or their combination. At 24 hours, only LPS at the high concentration (500 ng/mL) in the Caco-2 system increased the expression of PKM2 (data not shown). Increased incubation to 48 hours enhanced confluency in plates and resulted in a significant increase (~38%) of PKM2 expression.

To confirm the involvement of PKM2 in the inflammatory response, we evaluated its levels in the absence and presence of a novel anti-inflammatory molecule of flaxseed currently under

investigation, Linoorbitide-A (LOB-A). At 200 nM, LOB-A in the presence of LPS reduced mRNA levels of PKM2 by 41% compared to LPS alone (Figure 4.7-A). Consistent with genetic expression data, ELISA quantification was performed and showed that PKM2 levels decreased from 1.5 ng/mL in the presence of LPS alone to 1 ng/mL after LOB-A treatment (Figure 4.7-B).

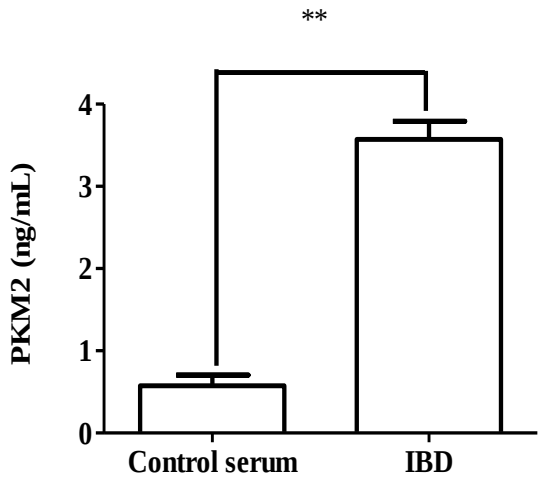

Figure 4.1 Mean ± SD serum PKM2 concentration in standard control (n=4) (from Biocell Laboratories) and newly diagnosed inflammatory bowel disease (IBD) patients (n=33) (Crohn's Disease, n=21 and Ulcerative Colitis, n=12). Concentrations were measured using a PKM2 ELISA kit. **p-value of two tailed t-test < 0.01.

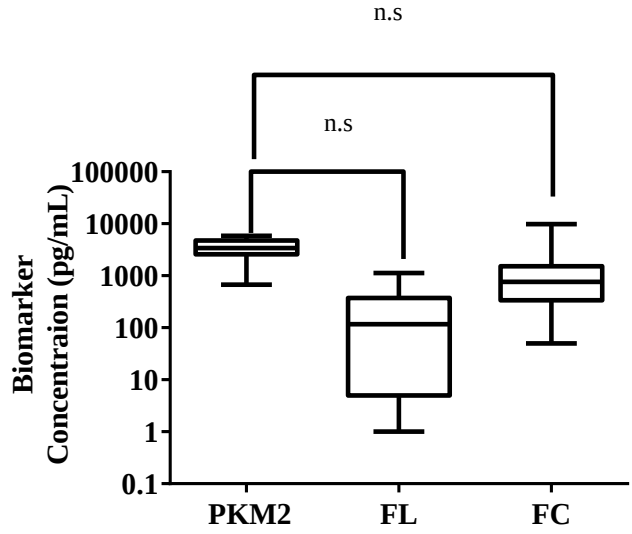

Figure 4.2 Boxplots with inter-quartile ranges comparing serum PKM2, fecal lactoferrin (FL) and fecal calprotectin (FC)were measured as biomarkers for IBD in 33 patients with newly diagnosed inflammatory bowel disease (IBD). n.s No significant difference in the interquartile ranges.

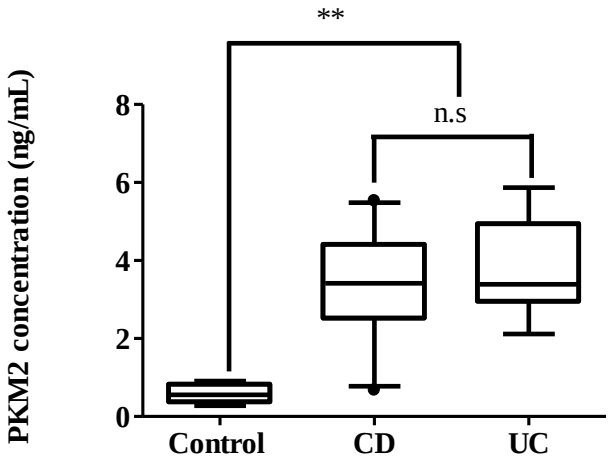

Figure 4.3 Serum PKM2 concentrations (ng/mL) in newly diagnosed patients with Crohn`s Disease (CD) (n =21) and Ulcerative Colitis (UC) (n= 12) (as determined by endoscopic evaluation) in comparison to control (n = 4) serum. Serum PKM2 was measured by a PKM2 ELISA kit. n.s No significant difference. **p-value of two tailed t-test < 0.01.

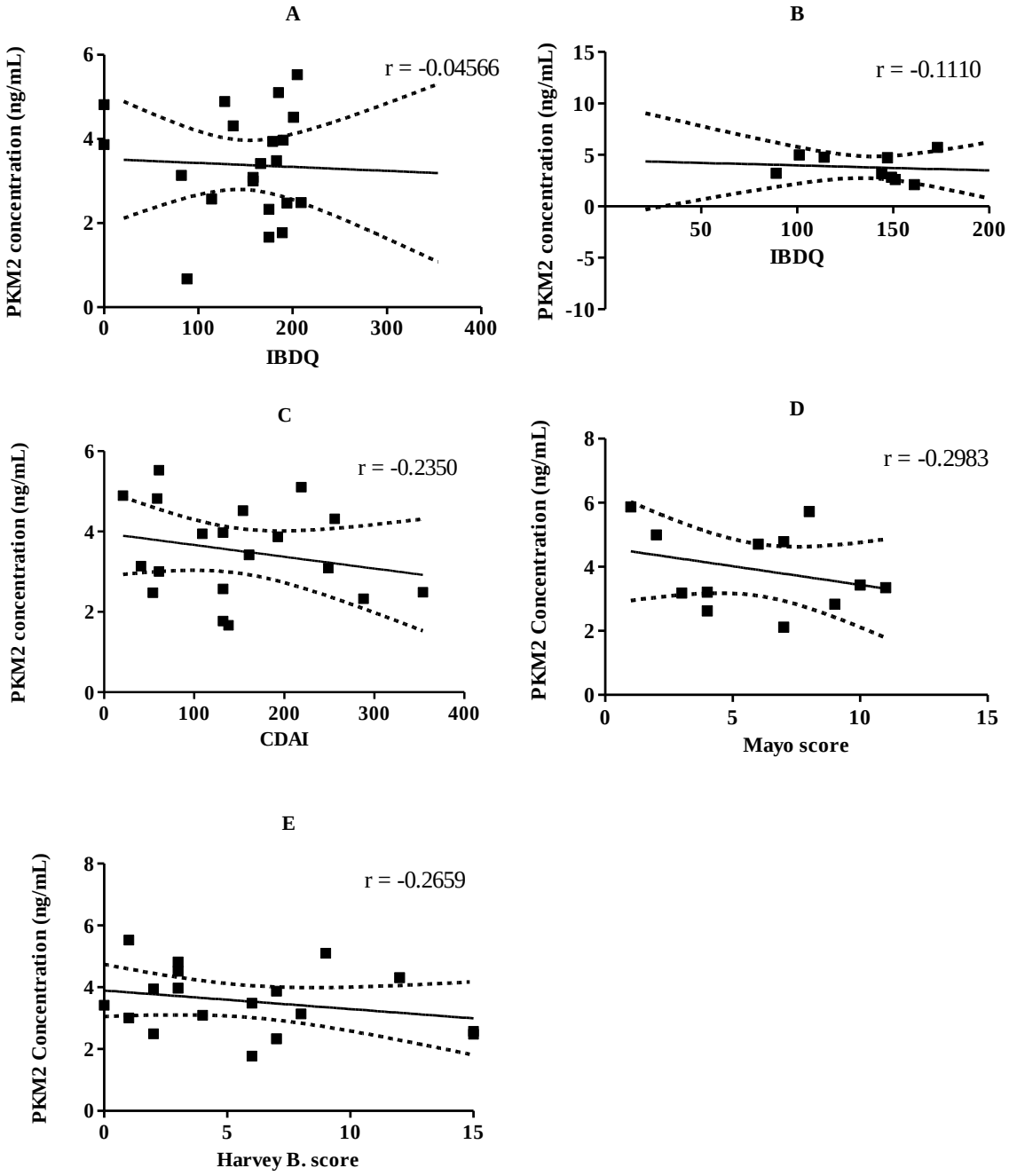

Figure 4.4 PKM2 serum concentrations (ng/mL) in correlation to disease activity scores in inflammatory bowel disease (IBD). Panel A represents IBD questionnaire (IBDQ) in Crohn`s disease patients. Panel B represents IBD questionnaire in Ulcerative Colitis patients. Panel C represents the dedicated Crohn`s disease activity index (CDAI) scoring system. Panel D represents the specialized Ulcerative Colitis Mayo scoring system. Panel E represents the Harvey B. scoring system. Data analysis used a two-tailed correlation with linear regression of 95% confidence intervals. The correlation was not significant at p<0.05.

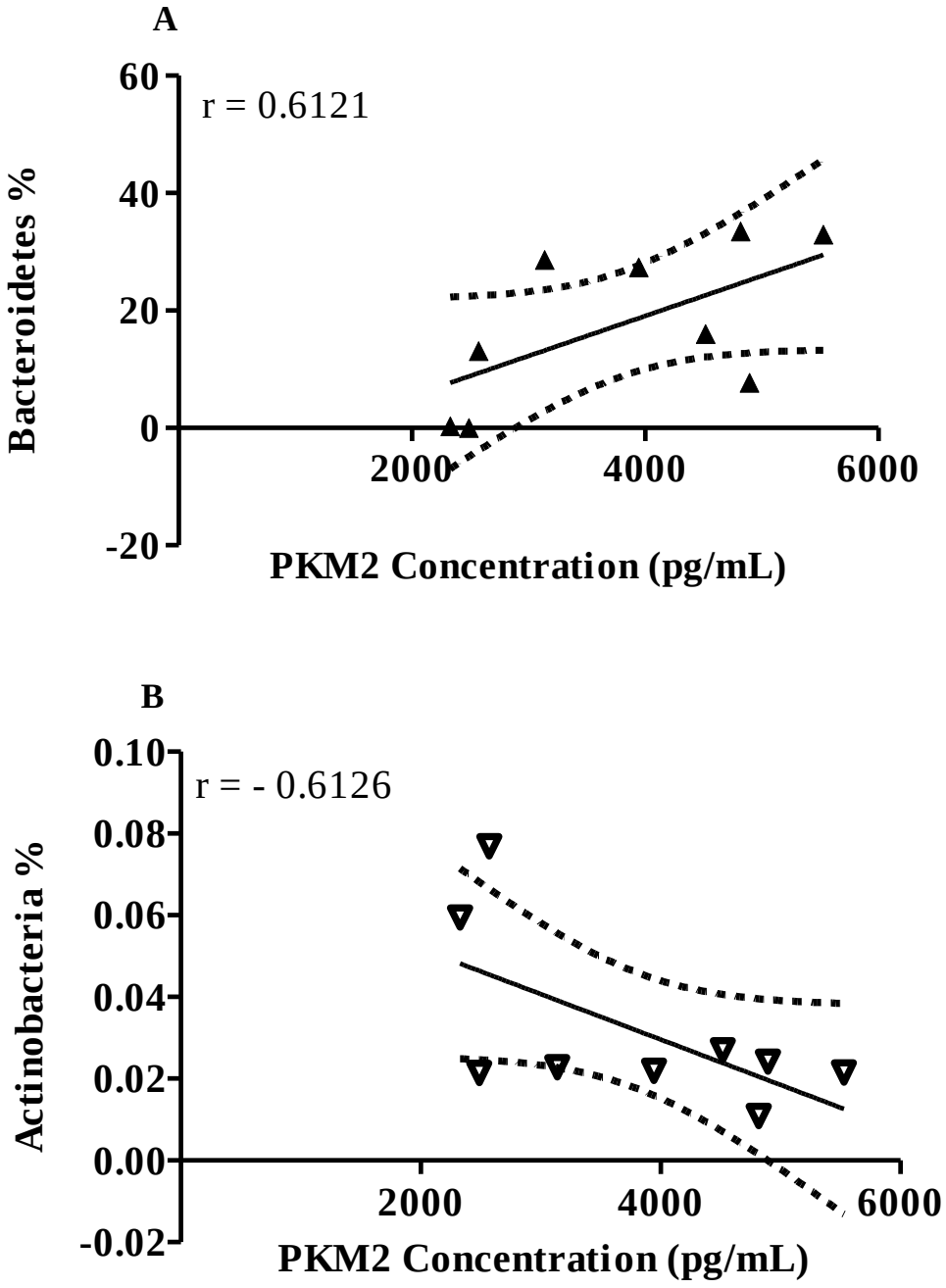

Figure 4.5 Correlation of PKM2 serum concentration (pg/mL) to normal flora (percentage of total bacterial content) in CD. Two tailed correlation was performed with linear regression of 95% confidence intervals. Correlations between serum PKM2 and Bacteroidetes and Actinobacteria were significant with a p-value <0.05.

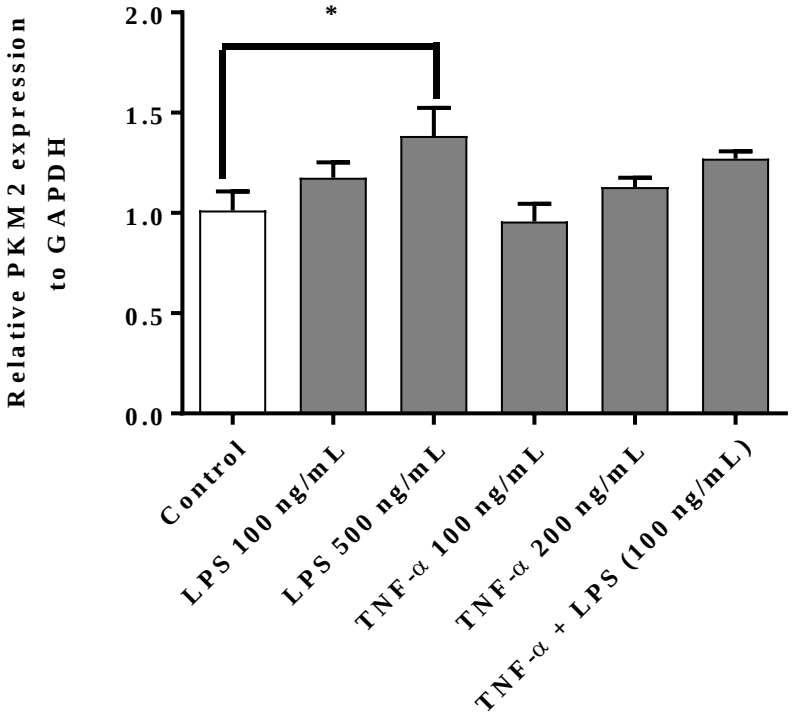

Figure 4.6 Relative PKM2 mRNA expression in of Caco-2 to housekeeping gene GAPDH. Total RNA was collected after 48 hours of incubation with different inflammatory inducers (LPS 100 ng/mL, LPS 500 ng/mL, TNF-α 100 ng/mL, TNF-α 200 ng/mL and (TNF-α + LPS 100 ng/mL)) and reverse transcribed to cDNA and amplified using qPCR. Data are presented as means ± S.D, n=3, * p-value < 0.05.

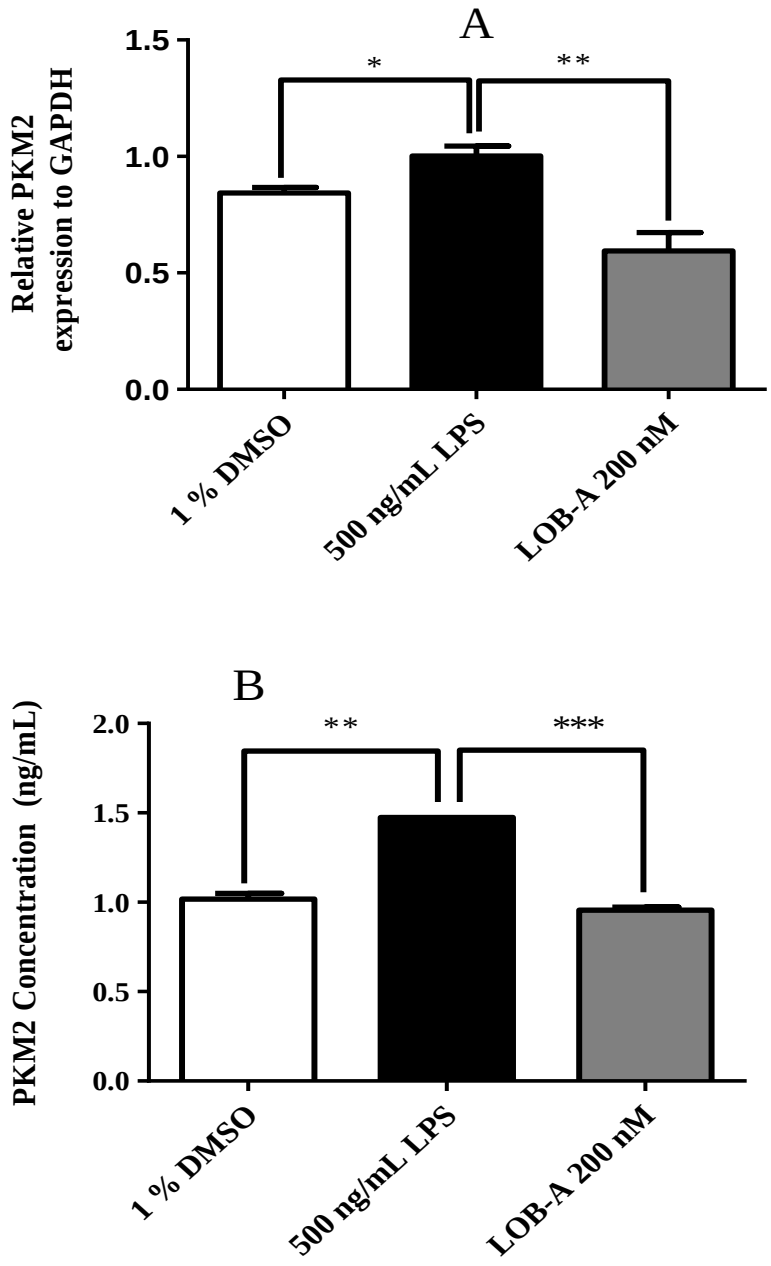

Figure 4.7 A- PKM2 genetic regulation upon exposure to LPS (500 ng/mL) for 48 hours. Expression of PKM2 mRNA relative to GAPDH mRNA in the presence of 500 ng/mL LPS. Data are presented as means ± S.D, n=3; one way ANOVA was performed and data were compared to LPS alone, * p-value < 0.05. B- ELISA-PKM2 concentration. Data are presented as means ± S.D, n=3, one way ANOVA was performed and data was compared to LPS alone, * p-value < 0.05,** p- value < 0.01 and , p- value < 0.001.***

4.4 Discussion

IBD is a chronic recurrent inflammation of the gastrointestinal tract of multifactorial origin[137]. Due to the high cost of treatment, morbidity, and high relapse rates[104, 137, 168, 186, 311], in-depth studies to discover biomarkers that can be used to assess disease progression and treatment effectiveness is a necessity. PKM2 is emerging as a hub between cellular energy consumption pathways and inflammatory processes[297, 304]. Therefore, an assessment of changes in PKM2 expression in IBD and with/without treatment will facilitate its utilization as a biomarker for disease, disease progression, and response to treatment. In this study, we assessed the use of PKM2 as a biomarker for IBD *in vivo* and *in vitro.* In addition, we assessed if treatment could alter this expression, and for a proof-of-principle for that concept we used LOB-A, which was shown to modulate inflammatory responses in our previous studies favorably. Through a combination of *in vivo* serum PKM2 protein expression analysis in newly diagnosed IBD patients and *in vitro* evaluation of PKM2, we identified that PKM2 could potentially serve as a biomarker in IBD. Lastly, PKM2 was predictive of dysbiotic gut microbiome in CD.

Our findings in newly diagnosed IBD patients showed that serum PKM2 was elevated in comparison to serum from healthy adults, but serum PKM2 levels could not differentiate between CD and UC. This observation is consistent with several reports where elevated faecal PKM2 helped differentiate between IBS and IBD, but not the sub-classification of IBD[312]. This could be due to the generalized involvement of PKM2 in inflammation and reduction of apoptosis with upregulation of this enzyme[305], which is a common feature of both UC and CD. Serum PKM2 was not significantly correlated with disease activity scores in CD and UC. This relationship was not significant, perhaps in part due to the small sample size, or the inherent variability in these disease activity scores.

In newly diagnosed IBD patients the elevated serum PKM2 showed limited interindividual variation, in contrast to the current biomarkers of IBD, FC and FL, which exhibited relatively large variability among this patient sample. This finding may be compared with Joshi *et al* who reported no changes in fecal PKM2 levels in three different age groups of healthy volunteers, while fecal samples of lactoferrin and calprotectin varied significantly across the age groups[313]. This low variability in serum PKM2 among IBD patients warrants further evaluation into its possible inclusion into the panel of biomarkers for IBD as it may improve accuracy and sensitivity of IBD diagnosis, disease progression, and or response to treatment[314-316]. The current biomarkers of IBD,

FC and FL, become elevated with activation and recruitment of macrophages, dendritic cells, and other immune cells in attempts to resolve inflammatory status[317]. However, both FC and FL are nonspecific as these molecules perform multifunctional roles associated with the inflammatory state and are observed in other chronic inflammatory conditions[11, 318, 195, 319]. Their lack of specificity and significant variation limits their effectiveness and, hence, the current biomarker panels fail to correlate strongly to endoscopy, "the gold standard" for IBD diagnosis[316]. The expansion of biomarker panels to capture multiple biological pathways can fortify the use of inflammatory biomarkers for disease diagnosis. Elevation of serum PKM2 captures a different aspect of the inflammatory process, where its increased expression in IBD may be the result of a sudden shift to increased metabolic rate and oxidative stress, which requires more efficient cellular ATP generation with reduced oxidative phosphorylation[297, 320]. Hence, PKM2 with its different functional role in inflammation, might add to the current biomarkers and collectively these biomarkers may improve accuracy of IBD diagnosis and disease progression.

Prior to its further evaluation as a putative biomarker in IBD and possible inclusion into a panel of biomarkers, measurement of serum PKM2 must address an important limitation of the current study. The use of a commercially available ELISA kit to estimate serum PKM2 concentration offered a convenient means to preliminarily investigate PKM2 in IBD patients. However, the available kit cannot differentiate between the different forms of PKM2, dimeric and tetrameric PKM2[321, 322]. These forms have different levels of endogenous activity, where the tetrameric form imparts the highest metabolic activity[302]. Therefore, advancing PKM2 utilization as a biomarker requires further analytical techniques and differentiation between isoforms to understand its role in IBD disease progression. Another important limitation is PKM2's critical role in glucose metabolism and inflammation and its abundance in several tissues including brain, muscles, small intestine, pancreas and astrocytes[323, 324]. This variable abundance in tissues with high glucose consumption asserts the need to select the sampling source for the use as an IBD biomarker. The collection of samples from both feces and blood circulation may better represent PKM2 arising from the IBD pathology, namely the intestinal epithelium that is shed in feces and the concomitant immune system activation presented both locally at the intestine and systemically. Lastly, statistical power analysis for recruitment of newly diagnosed IBD patients was performed, however, recruitment issues and patients' population in the city of Saskatoon limited the number

of participants involved. This potentially results in an underpowered study, where increasing study participants is necessary to increase the reliability of study power design.

Interestingly, in the patient subsample there was a positive correlation between serum PKM2 concentration and intestinal *Bacteroidetes.* The role of microbiota in IBD has been widely studied and correlated to its severity, prognosis and localisation[325-327]. Dysbiosis in IBD favors an 8-fold increase in the mucosal associated bacteria (aerobic and anaerobic) in patients with IBD in comparison to asymptomatic patients[170]. In CD, the gastrointestinal tract is leaky and potential penetration of microbiota to the systemic circulation is possible[328]. Appearance of *Bacteroidetes* in the systemic circulation could increase extraintestinal inflammatory symptoms, which could cause an indirect increase of serum PKM2. Further investigation that is focused on the CD population might reveal more details about PKM2 and its correlation to microbiota.

In conclusion, newly diagnosed IBD patients have elevated serum PKM2 levels, although PKM2 measured by ELISA cannot differentiate between the IBD phenotypes or disease localization. Its potential use as a biomarker, however, warrants further investigation as PKM2's involvement in inflammation and cellular energy homeostasis and its small interindividual variability suggests its robustness as a biological indicator of IBD pathology regardless of age or IBD phenotype. Therefore, PKM2 might be a beneficial player in a panel of biomarkers that can be used to fortify the selectivity and sensitivity of the currently available biomarkers of IBD.

CHAPTER 5

Enterolactone-glucuronide upregulates INSIG-1 to modulate cholesterol metabolism in Caco-2 cells

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Enterolactone-glucuronide upregulates INSIG-1 to modulate cholesterol metabolism in Caco-2 cells

Ahmed A. Almousa[1], Alister Muir[2], Ed S. Krol[1], Jane Alcorn[1*]

Transitioning rationale:

The previous chapters focused on the assessment of the local anti-inflammatory and antioxidative effects of LOBs and ENL effects on the intestinal epithelium and ability to maintain epithelial barrier integrity in two different *in vitro* models for application in IBD. The intestinal epithelium also plays an important role in cholesterol homeostasis. Despite positive correlations, reports on flaxseed effects on cholesterol modulation lack identification of mechanisms involved. One possible mechanism is modulation of cholesterol absorption at the intestine to explain the hypocholesterolemic effects associated with flaxseed consumption. Hence, a continuation to the main objective of this thesis then, this manuscript explored how flaxseed lignans might impart their hypocholesterolemic response at the level of the intestinal epithelium.

Contribution statement:

Ahmed Almousa contributed to this manuscript by designing the study, performing experiments, data acquisition, data analysis and manuscript drafting.

Abstract

Modulation of cholesterol metabolism is considered an integral part of risk factor reduction in cardiovascular disease. The increased tendency of using natural products in conjunction with prescribed medication heightens the importance of research and clinical trials in this area. Changes in cholesterol metabolism have been attributed to intake of lignans from several plant origins.

Evidence suggests that the lignan enterolactone (ENL), improves lipid profiles in hypercholesterolemic patients. However, the nature of this modulation is yet to be discovered. This study investigated the effect of ENL and its conjugated form ENL-Glucuronide (ENL-Gluc) on cholesterol metabolism. We measured the effect of both ENL and ENL-Gluc on cholesterol uptake in the Caco-2 intestinal cell line, in the presence of a cholesterol trapping molecule U-18666A. In addition, we screened for ENL and ENL-Gluc induced gene expression patterns. Results showed that treatment with ENL and ENL-Gluc reduced cholesterol uptake by 2.94 and 1.99-fold at 20 µM, respectively, in comparison to vehicle control of 1% DMSO. Furthermore, only ENL-Gluc. significantly upregulated Insulin Induced Gene-1 (INSIG-1) by 3.8-fold when compared to untreated control at *p-value* < 0.01. Western blot analysis confirmed gene expression results. These changes were also confirmed phenotypically by increased retention of fluorescing cholesterol (NBD-Cholesterol) in the endoplasmic reticulum as a function of increased INSIG-1 expression. In conclusion, glucuronide conjugation of ENL alters its pharmacological effect in regards to cholesterol metabolism, through increased expression of INSIG-1. Further investigation is necessary in more metabolically active cell lines including hepatocytes, and in assessment of downstream pathways of cholesterol synthesis.

5.1 Introduction

Hypercholesterolemia is a known risk factor for coronary heart disease. In high income countries, hypercholesterolemia reaches incidence levels of up to 50% of the population[329]. Statistics from the United States also show that less than half of individuals with high LDL-cholesterol (LDL-C) do not receive cholesterol lowering treatments[330]. For individuals who do not take hypocholesterolemic medications or fail to meet their target levels, increasing interest is turning towards natural products for the management of cardiovascular disease risk factors. Among those natural products with evidence of cholesterol reducing effects are the lignans, a

group of phenolic bioactives found in several plants like sesame, flax, oats, and nuts[331, 332]. The safe and effective use of lignans in the reduction of risk factors such as hypercholesterolemia, though, warrants a rigorous understanding of their mechanisms of action.

Lignans are a group of phenylpropanoid dimers, where the phenylpropane units are linked by a central carbon -C8- of their propyl side chains[333]. The consumption of foods rich in lignan components is generally linked to reduced circulating blood cholesterol, an attribute confirmed by administration of lignan-enriched products or purified lignans[2, 11, 12, 82, 334, 335]. The ability of lignans to reduce circulating cholesterol may involve several molecular factors like upregulation of PPAR-ɣ and LXR-α [336], key transcriptional regulators of fatty acid homeostasis, or upregulation of cholesterol efflux transporters ABCA1 and ABCG1, as noted with the lignans, arctigenin and sesamin[337, 338]. However, the exact mechanism through which lignans mediate reductions in circulating cholesterol remains largely unknown.

With oral consumption, lignans undergo further metabolism to the mammalian lignans, enterodiol and enterolactone (ENL), by the colonic microbiota[256, 339]. Generally, the epidemiological literature attributes the health benefits of lignans to ENL[44, 71, 230, 235, 340]. Interestingly, the mammalian lignans are susceptible to extensive enterohepatic recirculation. Both intestinal and liver cells actively metabolize the mammalian lignans to glucuronide or sulfate metabolites during their absorption process[219]. Hepatic biliary excretion of glucuronide or sulfate conjugates of the mammalian lignans results in the presentation of the conjugated moieties to the gastrointestinal tract mucosa. Gastrointestinal luminal beta-glucuronidases and sulfatases can act to release the conjugated molecule to restore the mammalian lignan, which then can subsequently undergo a repeated cycle of enterohepatic circulation. The extensive conjugative metabolism of the mammalian lignans and the enterohepatic recirculation process results in the prolonged and repetitive exposure of the gastrointestinal tract mucosa and liver to the conjugated metabolites of the mammalian lignans. Although an understanding of the pharmacology of the mammalian lignans has received attention, almost no study has evaluated their conjugated metabolites.

The objective of this study was to investigate a potential mechanism through which the mammalian lignan, ENL, and its glucuronidated metabolite, ENL-glucuronide (ENL-Gluc), might mediate the cholesterol reduction effects noted with lignan consumption. Although *in vitro* assessment of cholesterol metabolism is a challenging process, the amphipathic steroid U-18666A (Upjohn Co.) has become a highly versatile chemical tool to understand cholesterol uptake and

intracellular trafficking[341, 342]. We used U-18666A to evaluate the potential cellular and molecular responses upon exposure of the intestinal cell line, Caco-2, to ENL-Gluc and ENL with an aim to identify which lignan form exerts pharmacological activity and whether the active form modulates cholesterol uptake or intracellular trafficking. We demonstrate that the glucuronidated metabolite of ENL modulates cholesterol trafficking. This finding has substantive ramifications as very few glucuronide conjugates of drugs or natural products are known to exhibit pharmacological activity; glucuronidation generally is considered as a metabolic inactivation pathway. Yet, with oral consumption the conjugated metabolites of the mammalian lignans are the predominant circulating forms. Our results provide intriguing support for ENL-Gluc as the principal form mediating the hypocholesterolemic effects associated with lignan consumption.

5.2 Materials and methods

5.2.1 Chemicals and Reagents

Cholesterol Uptake Cell-Based Assay Kit was purchased from Cayman Chemical (Ann Arbor, MI, USA). Ezetimibe-D-glucuronide (Ez-Gluc) and insulin induced gene-1 (INSIG-1) antibody (sc-390504) were purchased from Santa-Cruz Biotechnology (Dallas, TX, USA). Lamin B1 antibody (66095-1) was purchased from Proteintech Group (Rosemont, IL, USA), and served as a housekeeping protein. ER-ID® Red assay kit was purchased from Enzo Life Sciences (Framingdale, NY, USA). Enterolactone (ENL), fetal bovine serum (FBS), 0.025% trypsin–EDTA, bovine serum albumin (BSA) and TWEEN-20 were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Glucuronated ENL was synthesized in our according to a previously reported protocol[232, 343]. Colorectal adenocarcinoma-C2BBe1 (Caco-2-BBe) and dimethylsulfoxide (DMSO) was acquired from American Type Culture Collection (ATCC) (Manassas, VA, USA). Fab2-Goat anti-mouse IgG (H+L) secondary antibody-HRP conjugate (A24512), Dulbecco's Modified Eagle medium (DMEM)-high glucose and penicillin-streptomycin (10,000 U/mL) were purchased from Thermo-Scientific (Waltham, MA, USA). T-75 flasks were from Corning™ (Pittsburgh, PA, USA). For microscopic imaging, a ZOE fluorescence microscope was used (Bio-rad, Hercules, CA, USA).

5.2.2 Cell culture

The Caco-2 cell line was cultured in DMEM-High glucose (0.3 g/L glutamine and sodium pyruvate) supplemented with 10% (FBS), 1% penicillin/streptomycin, and maintained at 37°C

with 5% CO_2 in a humidified atmosphere. Initially, cells were passaged twice after removal from liquid nitrogen, medium was changed every two days, and cells in T-75 flasks were passaged at 70-80% confluency using 0.25% trypsin-EDTA. All experiments were carried out at passage number 52-65.

5.2.3 Cholesterol uptake experiment

Caco-2 cells were seeded onto 96-well plates at a density of 10,000 cells/well in regular supplemented DMEM-high glucose media (10 % FBS and 1% streptomycin and penicillin), and left to attach overnight (200 µL). After attachment, medium was removed and new medium (180 µL) supplemented with 1% streptomycin and penicillin but no FBS was added in combination with the cholesterol uptake kit fluorescing compounds, NBD-cholesterol (20 µg/mL) and U18666A (1:1000 dilution) per kit manufacturer recommendation. Simultaneously, 20 µL of ENL and ENL-Gluc (2 and 20 µM) were added to the wells, and 20 µM EZ-Gluc was used as a control for cholesterol uptake inhibition and 1% DMSO as a negative control. All chemicals were dissolved in 100% DMSO and diluted to 1% DMSO upon treatment. All experiments were done in triplicate on 3 different occasions and no wells contained more than 1% DMSO. After 24 hours of co-treatment (lignans + NBD-cholesterol + U18666A), plates were centrifuged at 400 × g at room temperature for five minutes. The medium was aspirated and 100 µL of wash buffer provided with cholesterol uptake assay was added to each well. Fluorescence was measured using a Biotek Synergy HT microplate reader (Fisher Scientific, Canada) at excitation and emission wavelengths of 485 nm and 535 nm, respectively. On a different occasion, a similar procedure was performed but cells were imaged using a ZOE fluorescence microscope (Hercules, CA, USA). Concentrations used in this experiment were determined from EC_{50} values for Ez-Gluc, ENL, and ENL-Gluc reported in the literature[68,350].

5.2.4 Cholesterol and Endoplasmic reticulum co-localization

ER-ID® Red assay kit was used to demonstrate potential localisation of cholesterol upon the co-administration of ENL-Gluc and U-18666A in the endoplasmic reticulum (ER). Using the same cell culture conditions and protocol as above, at the end of the exposure period an ER dye and Hoechst nuclear stains were added and incubated per manufacturer recommendations, washed with the cholesterol assay buffer, and visualized under the ZOE fluorescence microscope.

5.2.5 Extraction of total RNA and quantitative RT-PCR

Caco-2 cells were treated with ENL or ENL-Gluc for 24 hours without the use U-18666A to eliminate its potential effect on cellular processes. Total RNA was extracted from cells cultured on 6-well plates using Trizol and Qiagen RNeasy mini kit (Toronto, ON, Canada) by following the manufacturer protocols. Total RNA was then reverse transcribed by abm® 5X All-In-One RT MasterMix to cDNA (abm, Richmond, BC, Canada), and then gene expression was quantified using Power Sybr® Green PCR mastermix (Burlington, ON, Canada). The polymerase chain reaction was carried out using a one-step method, where initial incubation with reverse transcriptase took place at 42ºC for 30 minutes, followed by denaturation at 95ºC for 3 minutes, and after denaturation 40 cycles of annealing and extension performed for 15 seconds and 30 seconds each, with a final dissociation curve per manufacturer setting. This measurement was performed using an Applied Biosystems® 7300 system (Foster City, CA, USA). Primer sequences were designed using Integrated DNA Technologies Primer-Quest tool (https://www.idtdna.com/Primerquest/Home/Index) (Table 5.1). The $\Delta\Delta C_T$ method was used to determine the fold change in genetic expression using GAPDH as a housekeeping gene.

Table 5.1. Forward and reverse primer sequences for select genes involved in cholesterol transport and metabolism. Primers were designed using Primer-Quest tool on the IDT website.

Gene Symbol*	Forward primer	Reverse primer
INSIG-1	CTTGACTTTAGCAGCCCTATCT	CGTGATCAGCGTAGCTAGAAA
SREBP-1	CACTGAGGCAAAGCTGAATAAAT	TAGGTTCTCCTGCTTGAGTTTC
NPC1	CTAAACTTCTCCCAGCCTCTTC	GGATCACATTCACAGCCATCTA
LXR	CGATCGAGGTGATGCTTCTG	GGCAAAGTCTTCCCGGTTAT
CYP7A	CGGACAGCTAAGGAGGATTTC	GTCAAAGGGTCTGGGTAGATTT
NPC1L1	AGCACCTTGGTCATGGATTAG	TGGCAGCAACAGCCTTAATA
CETP	CTTCAACACCAACCAGGAAATC	CAGGAGATCTTGGGCATCTT
ABCA1	CCTGTCATCTACTGGCTCTCTA	CAGATTGGTGGAGGACACATAG
GAPDH	CAAGAGCACAAGAGGAAGAGAG	CTACATGGCAACTGTGAGGAG

*INSIG-1: Insulin induced gene-1, SREBP-1: Sterol receptor element binding protein-1, NPC1: Niemann-Pick Disease, Type C1, LXR: Liver X Receptor, CYP7A: cholesterol 7-alpha-

hydroxylase, NPC1L1: Niemann-Pick C1-Like 1, CETP: Cholesterylester transfer protein, ABCA1: ATP-binding cassette A1 and GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

5.2.6 Western blots of INSIG-1

Caco-2 cells were seeded in T-25 flasks at $1x10^6$ cells/mL density, and left to attach for 24 hours. Treatment with various concentrations of ENL-Gluc (2 and 20 μM) and 1% DMSO vehicle control was carried out for another 24 hours. Cells were lysed for protein extraction using the radioimmunoprecipitation assay buffer (RIPA) from Millipore (Billerica, MA, USA) in ice-cold conditions, where linearity of protein concentrations was verified. Protein concentrations were determined using Pierce™ BCA Protein Assay Kit (Rockford, IL, USA). Subsequently, 20 μg of total protein were transferred for gel electrophoresis using 10 % Mini-Protean Gel (Bio-Rad Technologies) at 120 volts for 15 minutes then 70 volts for 60 minutes. Transfer took place on a nitrocellulose membrane at 300 mA for 2 hours. Membrane blocking was carried out using 5% BSA in PBS for one hour. All primary antibodies (INSIG-1 and Lamin-B) (1:1000) were incubated overnight at 4°C in PBST and then incubated with the secondary antibody (1:3000) for 2 hours at room temperature. The blots were developed using enhanced chemiluminescence detection system (Novex® ECL) from Life Technologies (Burlington, ON, Canada). Images were taken using the AlphaImager™, and analyzed using AlphaView software through the band analysis module (San Jose, CA, USA). INSIG-1 protein band intensity was normalized to its corresponding housekeeping protein Lamin B intensity.

5.2.7 Statistical Analysis

All data were reported as means ± standard deviation (SD). Effects of treatments relative to controls were evaluated by using one-way ANOVA followed by Dunnett's post-hoc test. Differences were considered significant at $p\text{-}value < 0.05$. All data analyses were performed using GraphPad Prism 5 software (San Diego, CA, USA).

5.3 Results

5.3.1 Simultaneous incubation with U-18666A to study the ENL and ENL-Gluc effects on cholesterol metabolism

To understand the potential differential effect of ENL and ENL-Gluc on cholesterol metabolism simultaneously by trapping cholesterol, our initial experiments showed that when using U-18666A alone as a positive control for cholesterol uptake no significant difference was observed with the

corresponding NBD-cholesterol (negative control) wells in the Caco-2 cell line (data not shown). Therefore, the experimental design was adjusted where U-18666A was co-incubated with ENL and ENL-Gluc in order to study their effect on cholesterol localization and the potential mechanism for modulation of cholesterol homeostasis. Ezetimibe-glucuronide, used as a positive control, verified the plausibility of this experimental design as its co-incubation with U-18666A resulted in a significant reduction (56%) in fluorescence associated with tagged cholesterol (NBD). Unconjugated ENL reduced NBD-cholesterol fluorescence by 39.9% and 65.9% at 2 and 20 µM ENL, respectively (Figure 5.1). Similarly, ENL-Gluc showed reduction in NBD-cholesterol fluorescence when compared to control by 58.9% and 49.7% for 2 and 20 µM, respectively.

When visualized by fluorescence microscopy, both ENL and ENL-Gluc at 20 µM showed reduction in NBD-cholesterol fluorescence, but more reduction was observed in the unconjugated form as seem in Figure 5.2. Interestingly, ENL-Gluc showed clusters of concentrated NBD-cholesterol, which were not observed in ENL and Ez-Gluc and the distribution of NBD-Cholesterol was considerably different from ENL and Ez-Gluc.

5.3.2 Effect of ENL and ENL-Gluc on INSIG-1 and SREBP expression.

To test for the potential mechanism involved in ENL and ENL-Gluc regulation of cholesterol trafficking, we assessed the genetic expression of a number of proteins important in cholesterol homeostasis namely INSIG-1, SREBP-1, NPC1, LXR, CYP7A, NPC1L1, CETP and ABCA1. Among the genes tested, only INSIG-1 and SREBP-1 showed significant differences in mRNA levels with exposure to ENL and ENL-Gluc. In particular, ENL-Gluc upregulated INSIG-1 by 1.9 and 3.8-fold at 2 and 20 µM, respectively (Figure 5.3A). ENL increased INSIG-1 expression (insignificantly) by 1.4 and 2.06 fold at 2 and 20 µM, respectively for the same duration of incubation (Figure 5.3C). Additionally, SREBP-1 was significantly downregulated by ENL-Gluc (0.45 fold of the control), while ENL did not influence the regulation of SREBP (Figure 5.3B and D). Western blot analysis further confirmed upregulation of INSIG-1 at the protein level in Caco-2 cells exposed to ENL-Gluc (1.4-fold at 2 µM (insignificant) and 1.8-fold at 20 µM ENL-Gluc) (Figure 5.4).

5.3.3 NBD-Cholesterol retention in the endoplasmic reticulum

To visually localize and understand the distribution of NBD-Cholesterol, we tagged the endoplasmic reticulum (ER) with a specific dye (ER-ID®) to verify the co-localization with

cholesterol upon administration of ENL-Gluc at 2 and 20 µM. As can be seen in Figure 5.5, increased accumulation of NBD-cholesterol was observed with increased concentrations of ENL-Gluc.

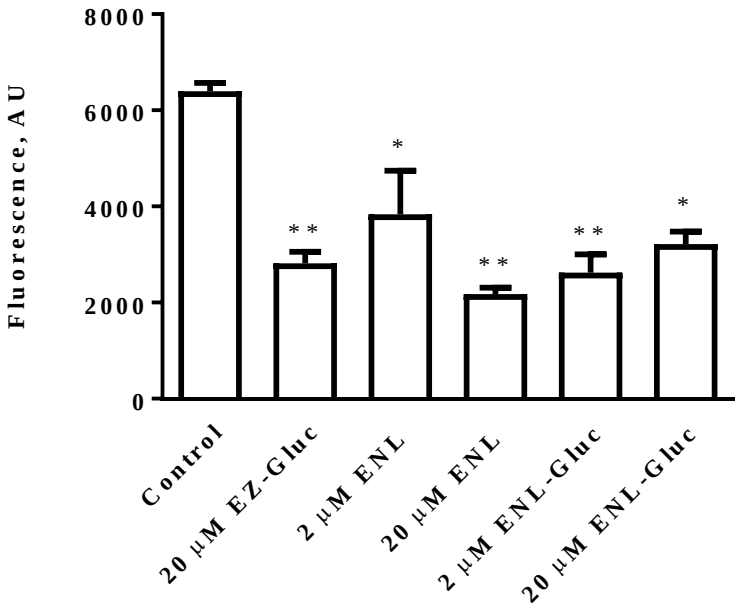

Figure 5.1 NBD-Cholesterol fluorescence in Caco-2 cell line after 24 hours of treatments with enterolactone (ENL) and enterolactone glucuronide (ENL-Gluc) and U18666A (cholesterol trapping molecule). Control represents NBD-cholesterol and U18666A alone. Positive control was achieved by using 20 µM EZ-Gluc (cholesterol absorption inhibitor) with NBD-cholesterol and U18666A. Fluorescence was measured using a Biotek Synergy HT microplate reader. Data are presented as means ± S.D, n=3, * p-value < 0.01, ** p- value < 0.001.

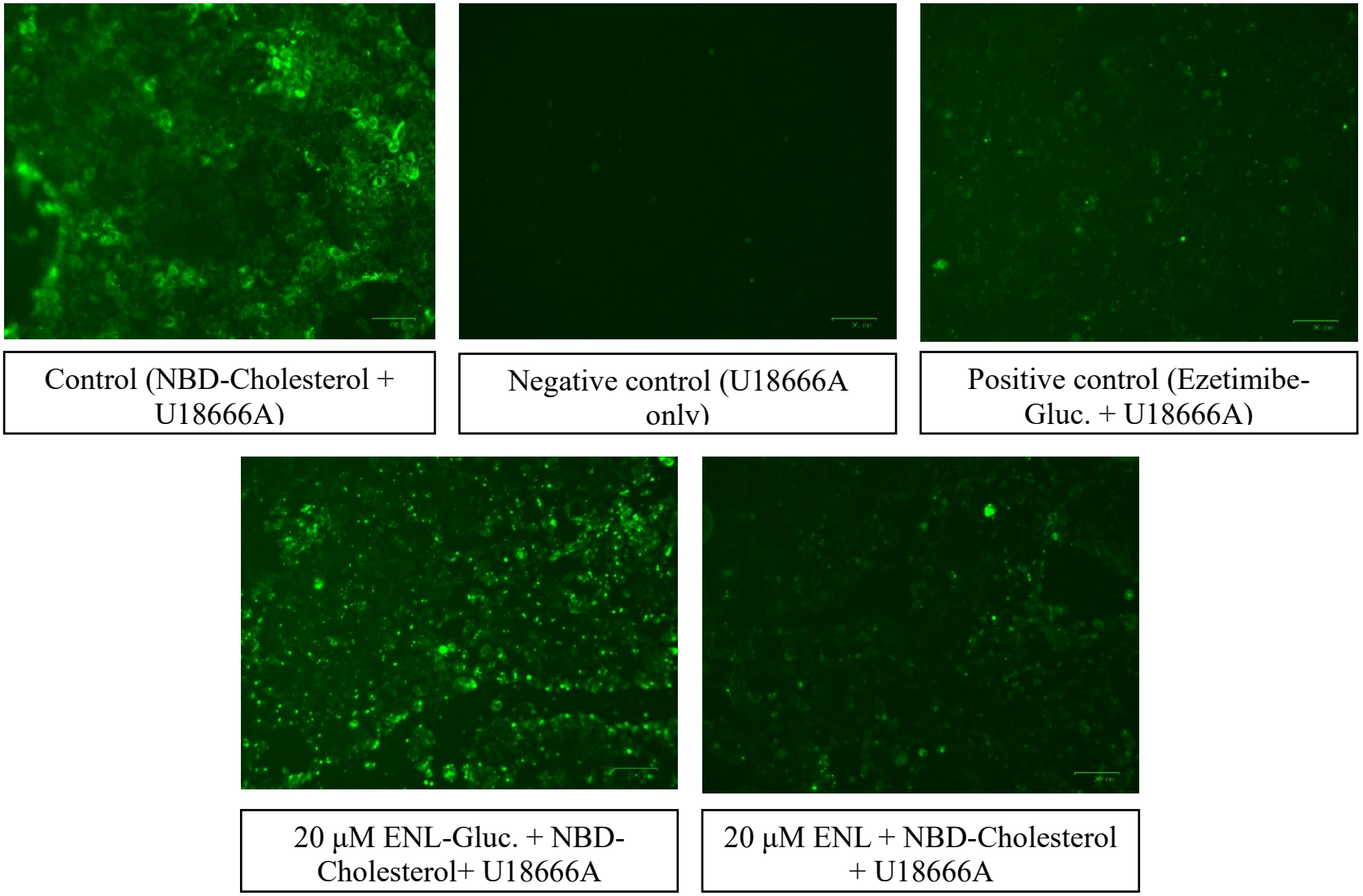

Figure 5.2 Images of NBD-Cholesterol fluorescence in Caco-2 cells after 24 hours of treatments with enterolactone (ENL) and enterolactone glucuronide (ENL-Gluc) and U18666A (cholesterol trapping molecule). Control represents NBD-cholesterol and U18666A alone; Negative control was U18666A; Positive control was achieved by using 20 µM EZ-Gluc (cholesterol absorption inhibitor) with NBD-cholesterol and U18666A. Images were taken using a ZOE fluorescence microscope.

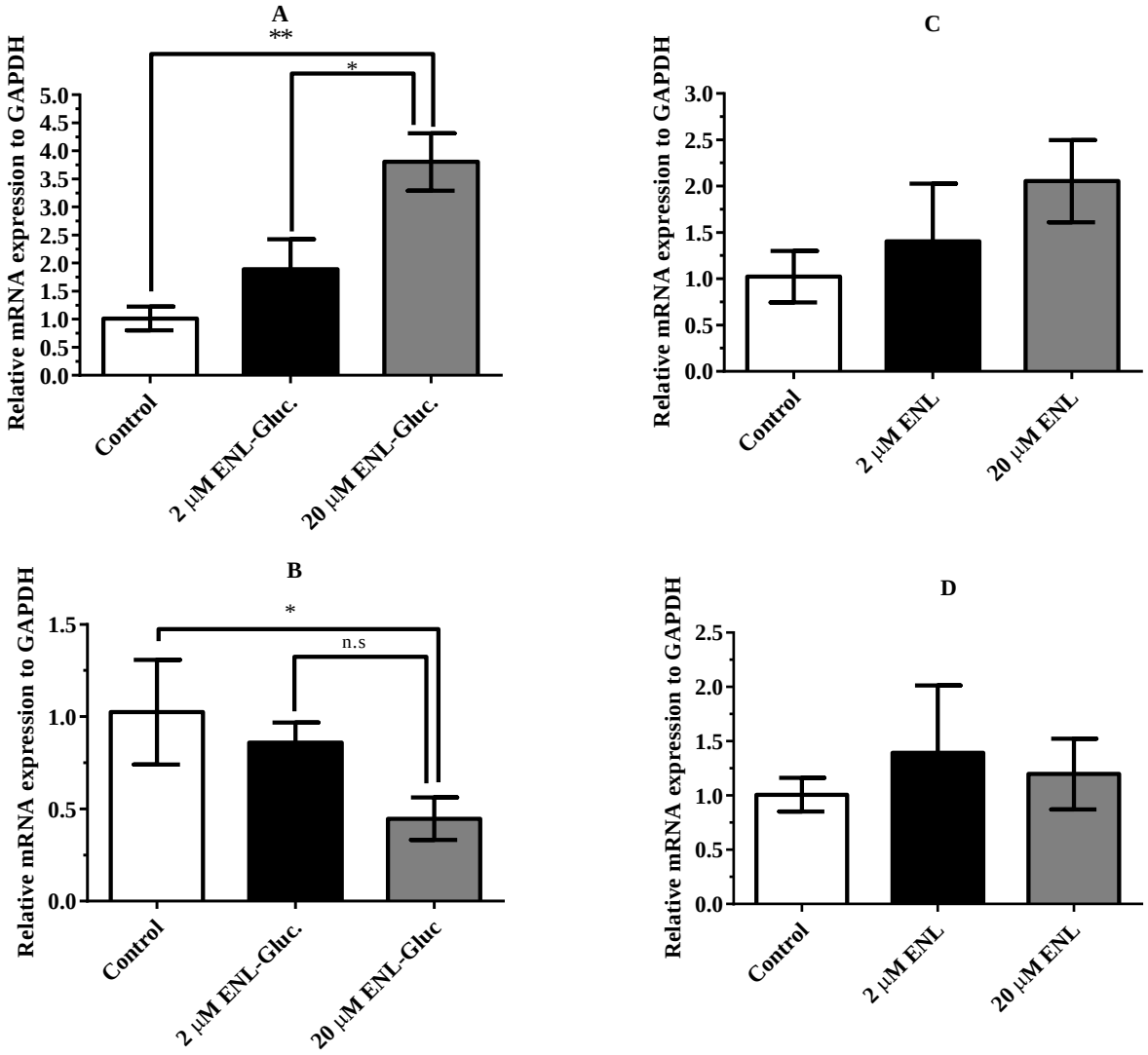

Figure 5.3 Mean mRNA expression ± S.D of INSIG-1 and SREBP-1 in Caco-2 cell line after treatment with 2 and 20 µM enterolactone (ENL) and enterolactone glucuronide (ENL-Gluc) without U18666A. Controls were treated with 1% DMSO. A- INSIG-1 expression after 24 hours ENL-Gluc treatment. B- SREBP-1 expression after 24 hours ENL-Gluc treatment. C- INSIG-1 expression after 24 hours ENL treatment and D- SREBP-1 expression after 24 hours ENL treatment. Data are presented as means ± S.D, n=3, * p-value < 0.05, ** p- value < 0.01.

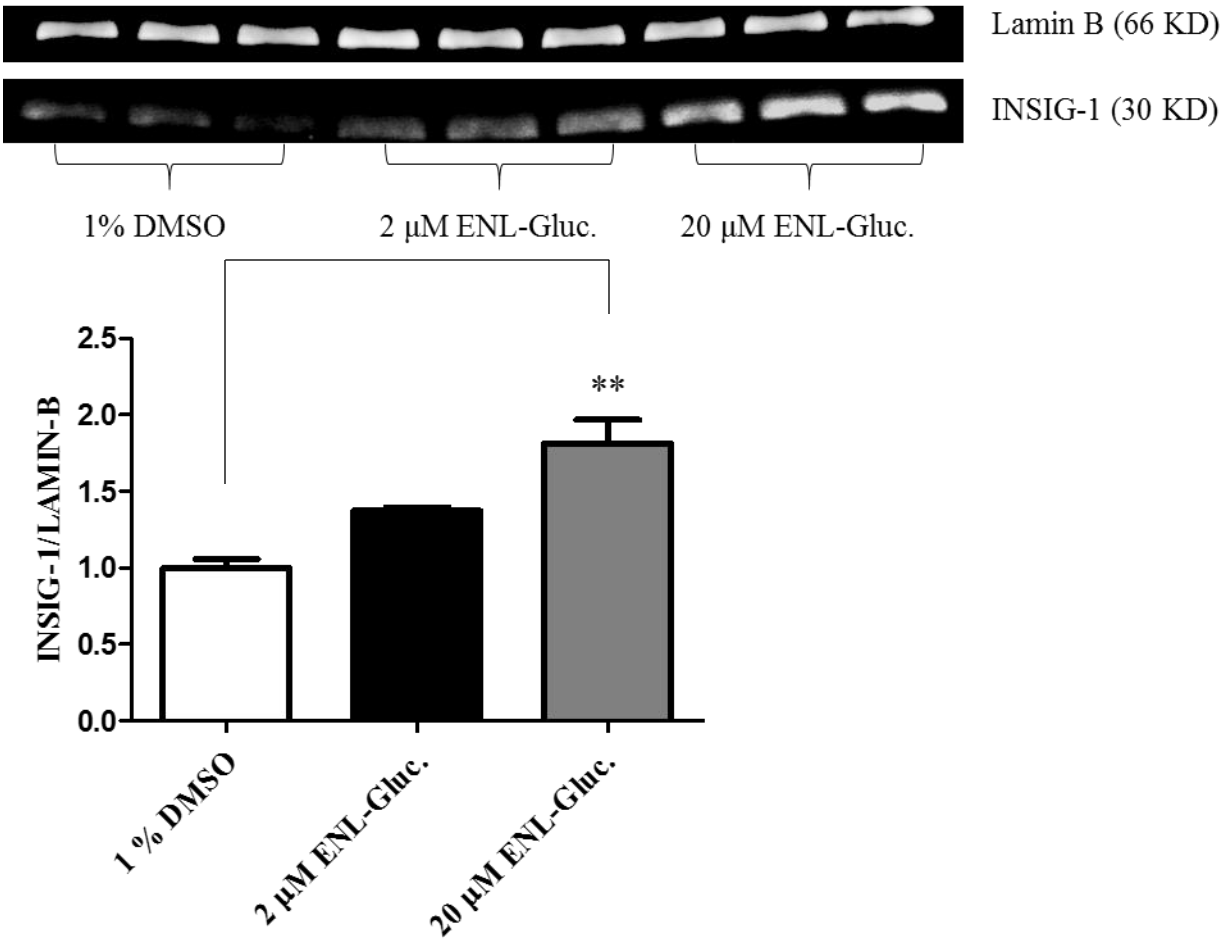

Figure 5.4 Protein expression of INSIG-1 in Caco-2 cell line after 24 hours treatment with 1% DMSO (control), 2 and 20 µM ENL-Gluc. Lamin B served as a housekeeping protein and was used to normalize band intensity at each corresponding band. Images were taken using the AlphaImager™, and analyzed using AlphaView software through the band analysis module (San Jose, CA, USA). Data were presented as means ± S.D, ** p- value < 0.01.

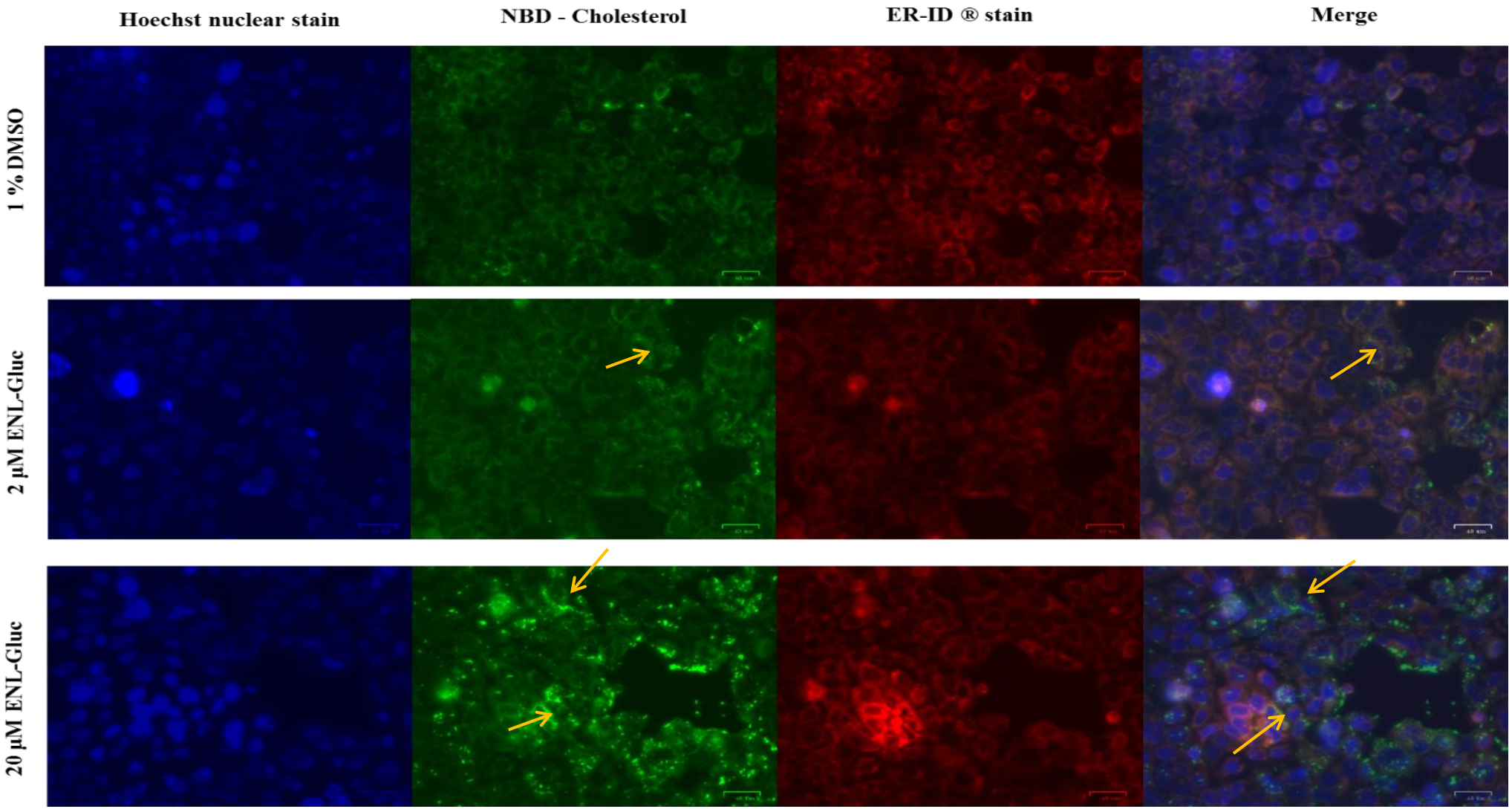

Figure 5.5 Distribution of NBD-cholesterol within the endoplasmic reticulum with different ENL-Gluc concentrations. Caco-2 cells were treated with 1% DMSO, 2 and 20 µM ENL-Gluc for 24 hours simultaneously with U18666A and NBD-Cholesterol. Staining used ER-ID® for ER, Hoescht for nuclear stain, and NBD tagged cholesterol. Images were taken using a ZOE fluorescence microscope at 10X magnification.

5.4 Discussion

An understanding of the pharmacological mechanism of action of natural products is becoming increasingly important for evidence based decision making on their utility in clinical practice. Such understanding will help to identify the nature of usage, whether it is chemopreventive or therapeutic, and identify potential interactions or potentiation of effects with currently approved therapeutics. Unlike regular therapeutics, natural products are often mixtures of several components with significant differences in their chemistry and biological activities, suggesting the importance of studying individual components of a natural product. The mammalian lignan, ENL, arises from a number of different precursor plant lignans, and has been associated with improved cholesterol levels, reduced tumor growth, improved glycemic control, and amelioration of inflammation[3-5, 8, 9, 70, 75, 344]. The mechanism through which ENL improves cholesterol levels in interventional studies, remains unclear.

Cholesterol in the intestine is supplied from three sources; diet, circulating bile and intestinal epithelium sloughing. Cholesterol from these sources is taken up by intestinal cells and is either effluxed again (ABCG5/8) to the lumen or sequestered in the form of chylomicrons to be transferred to the basolateral side of the polarized intestinal epithelium[345]. Cholesterol molecules are absorbed passively through the formation of micellar structure. More recently, an apical protein receptor, the NPC1L1 was identified to facilitate cholesterol absorption. Inhibition of NPC1L1 is attributed to be Ezetimibe's mechanism of action to lower total cholesterol levels in the blood. Studies also suggest that NPC1L1 is the rate limiting step for cholesterol uptake. Other scavenger receptors might be involved in cholesterol uptake including scavenger receptor type B (SR-BI), but these are thought not to be as critical as NPC1L1[346].

In this work we investigated the effect of the mammalian lignan, ENL, and its glucuronic acid conjugated form, ENL-Gluc, on cholesterol metabolism in the intestinal cell in an effort to understand the underlying mechanism of action. Similar to ezetimibe that requires glucuronidation to inhibit NPC1L1, we speculated that ENL would require a similar mechanism.

We used the combination of the Caco-2 intestinal epithelium cell line with the cholesterol trafficking inhibition molecule, U18666A, and fluorescing NBD-cholesterol to assess cholesterol uptake and metabolism. This is an increasingly used *in vitro* system for assessment of the effect of new chemical entities on cholesterol homeostasis[347]. In the hepatocyte, studies show the ability of phytoestrogens, such as genistein and daidzein, to favourably modulate cholesterol homeostasis

through mechanisms involving inhibition of cholesterol synthesis, inhibition of esterification and reduced expression of LDL-receptors[348]. As well, ENL is reported to upregulate the LDL receptor in hepatocytes[349] suggesting this as a potential mechanism for reduced cholesterol levels. However, our understanding that ENL undergoes extensive phase II metabolism (particularly glucuronidation) and enterohepatic recirculation is suggestive of ENL and ENL-Gluc presence at the intestinal mucosal layer.

Both ENL and ENL-Gluc caused reductions in the amount of fluorescing cholesterol within the intestinal cell similar to the FDA approved cholesterol absorption inhibitor prodrug, ezetimibe. In our experiments, we used a system that enhances cholesterol accumulation (U18666A) within the cell (only if it gets absorbed) and simultaneously added ENL or ENL-Gluc. This system allowed differentiation between cholesterol absorption inhibitors like Ez-Gluc, as seen in Figure 5.2 where NBD-cholesterol is almost absent intracellularly, and cholesterol metabolism modulators, which allows the uptake of cholesterol to the cell, but imparts their effect through altered cholesterol trafficking. This system showed that ENL and ENL-Gluc both reduced the concentrations of NBD-cholesterol as seen in Figures 5.1 and 5.2. But we observed different intracellular accumulations in the case of ENL-Gluc, where cholesterol was retained in the ER and its trafficking was altered. Upon further investigation, we found that ENL-Gluc significantly upregulated INSIG-1. Although it was not significant, ENL gave a trend toward upregulation of INSIG-1, an observation that could be explained by the initiation of glucuronidation of ENL in the Caco-2 cell line. Previously, our laboratory demonstrated the ability of Caco-2 cells to mediate the rapid metabolism of ENL to glucuronic acid conjugates with complete metabolism within 24 hours of incubation[221]. Conventionally, glucuronidation is a drug deactivation pathway, but in some instances, it results in the activation of parent drugs like ezetimibe or production of an active metabolite in the case of morphine[350, 351]. Similarly, ENL glucuronidation seems to be involved in the activation of ENL and its potential role in cholesterol homeostasis. Typically, glucuronidation takes place intracellularly, mainly within the endoplasmic reticulum membrane[352], and it either interacts with the target receptor or is effluxed to exert its effect through a signaling pathway that is currently unknown.

INSIG-1 upregulation has a role in inhibition of lipogenesis in adipocytes and reduction in risk of coronary heart disease[353-355]. Under cholesterol rich conditions, INISIG-1 acts as an anchor protein to trap sterol regulatory element-binding proteins and its cleavage-activating protein

(SREBP-SCAP) in the endoplasmic reticulum and prevent their dissociation[356, 357]. Thus, SREBP cannot translocate to the Golgi for proteolytic activation and further to the nucleus to activate cholesterol synthesis and fatty acid synthesis[358]. The observed SREBP-1 downregulation is correlated with a possible reduction in cholesterol synthesis as well as potential treatment for fatty liver disease[359]. In addition, INSIG-1 upregulation considerably increases the sensitivity of cells to sterol-mediated feedback inhibition of cholesterol synthesis[360], suggesting fortified activity at increased levels of cellular cholesterol. This is consistent with clinical reports that showed a beneficial effect in hypercholesterolaemic patients[11, 82] but not in the healthy population[98]. In addition to trapping SCAP complex in the ER and preventing further cholesterol synthesis, INSIG-1 upregulation accelerates the degradation of HMG-CoA reductase conferring an effect similar to statins[361]. These combined effects of INSIG-1 make this gene a plausible target for cholesterol metabolism as a hub in cellular metabolism[358]. Reports on cholesterol modulation by the lignan, sesamin, is restricted to downregulation of SREBP-1, or upregulation of the cholesterol efflux transporters (ABCA1 and ABCG1)[338, 362]. However, involvement of the INSIG-1 pathway was not explored to explain the cholesterol modulating effects of sesamin. Upregulation of INSIG-1 represents a novel finding for lignan modulation of cholesterol homeostasis.

In conclusion, we are the first to report the possible hypocholesterolemic mechanism of action for the mammalian lignan, ENL, which involves upregulation of INSIG-1 with concomitant alteration in intracellular cholesterol trafficking. Furthermore, it appears that the glucuronide metabolite is the active form that mediates this effect. Further investigations are necessary to understand the fate of enhanced cholesterol trafficking to the ER and to confirm this potential mechanism of action in appropriate in vivo models of hypercholesterolemia.

CHAPTER 6

GENERAL DISCUSSION

6.1 Discussion

Translational health research that connects clinical practice to benchtop sciences shall not be exclusive to conventional therapeutics, rather it should be extended to natural health products and alternative medicine. Unlike conventional therapeutics a plethora of natural products are available in the market without significant correlation to health benefits and lack of support to claims typically coming from allopathic health care providers, the internet, and by word of mouth[363]. Reportedly, this is a natural human inclination toward products obtained from mother nature and perceived as a safe and sometimes effective alternative to conventional medicine[364]. This public behavior leaves the health care professional and translational health researcher with significant responsibility to enhance scientific evidence available for clinical utilization of natural products[365]. Therefore, in depth safety and pharmacological behavior evaluations are becoming a necessity to aid clinical recommendations by physicians, pharmacists and other health care professionals. Flaxseed bioactives are among those natural products that require translational verification of specific pharmacological activities.

My laboratory has been focused on flaxseed natural product safety, tolerability, metabolism, and mechanism of action[12, 22, 51, 206, 217, 221, 232]. Current knowledge of flaxseed extracts reveals a strong association in several chronic diseases like cancer, diabetes, high blood pressure and inflammatory disorders[2-7]. This association can be attributed to significant anti-inflammatory activity, immunosuppressant potential, antioxidant activity and sex hormone modulating effects[9, 44, 47, 366]. However, the current understanding of distinct flaxseed lignan pharmacokinetic properties shows reduced oral bioavailability and susceptibility to enterohepatic circulation[257]. In addition, flaxseed cyclic peptides (LOBs) have high molecular weights that theoretically preclude their ability to permeate through the intestinal epithelium upon oral intake[244]. Based on these pharmacokinetic observations and reported pharmacological activity, we proposed the potential utilization of flaxseed bioactives (lignans represented by ENL and LOBs) to impart local effects on the intestinal epithelium. Specifically, our interest focused on inflammatory bowel disease (IBD) and hypercholesterolemia, where intestinal epithelium is crucially involved in their pathogenesis and progression[116, 168, 367, 368]. Among the flaxseed bioactives we focused on ENL, LOB-A, LOB-J and LOB-ACEJ, as limitation in resources (time, money, workforce and purified

individual components in the case of LOBs) precluded the assessment of other bioactives. In addition, we focused on these LOBs due to the distinct cytotoxicity profile differences between LOB-A and LOB-J, where LOB-A is significantly more toxic at lower concentrations (5 µM when compared to LOB-J). Indeed, observation of positive findings with the examined compounds will warrant further evaluation of other lignans and LOBs. We performed several *in vitro* experiments including effects on barrier integrity, potential immune regulators, oxidative stress, biomarker verification and cholesterol metabolism to verify the proposed benefits in IBD and hypercholesterolemia.

The intestine plays a critical role in cholesterol metabolism. On its apical side (intestinal lumen), cholesterol molecules are absorbed either passively through the formation of micellar structure or through Niemann-Pick C1-Like 1 (NPC1L1) receptor which was identified to facilitate cholesterol absorption[369]. Intracellularly, cholesterol in the intestine is supplied from three sources; diet, circulating bile and intestinal epithelial cell sloughing. Cholesterol from these sources is taken up by intestinal cells and is either effluxed to the lumen or sequestered in the form of chylomicrons to be transferred to the basolateral side of the polarized intestinal epithelium and then to systemic circulation[345]. These functions render the evaluations of ENL and LOB effects on cholesterol metabolism and absorption important to explain the hypocholesterolemic effect noted with the use of BeneFlax® (a flax lignan enriched complex) in a randomized clinical trial of hypercholesterolemic Chinese participants[11]. These studies warranted the need for investigations into how the lignans caused the hypocholesterolemic effects. The use of Caco-2 is an appropriate system to evaluate this as evidenced from studies that use this system in the literature[370]. In addition to its role in cholesterol metabolism and trafficking, the intestine plays a major role in IBD through several immune functions. These functions are presented through the maintained barrier integrity, modulation of oxidative stress and inflammation[137]. Therefore, an *in vitro* system that is simple yet capable of removing confounding effects is critical to begin understanding how ENL and LOBs may favorably modulate these key functions.

In our pursuit to verify the effect of flaxseed bioactives on IBD, we initially assessed their effect (LOBs and ENL) on intestinal barrier maintenance. Significant barrier loss and leakage was observed in subjects with active disease, rendering its maintenance a prime goal for the development of treatments and preventive measures. Hence, we used an *in vitro* system of HCT-8 (Chapter 2) cell line that mimics the intestinal epithelium and left it to polarize for eight days to

create distinct apical and basal membranes on a transwell system. This approach simulates intestinal lumen on the apical side and lamina propria on its basal side (where immune cells are typically concentrated). Other *in vitro* or *ex vivo* systems available include Ussing chambers, which mounts a graft of intestinal epithelium on a dynamic electrolyte flow device allowing enhanced representation of transporters and other cellular components when compared to an immortalized cell line (HCT-8)[371]. Another potential cell line that can be used is Caco-2, which requires longer incubation to achieve proposed polarization (14-21 days)[277, 372]. With the goal of conducting an initial screen, we used the HCT-8 cell line for rapid polarization patterns and proof of concept.

With the HCT-8 cell line we observed different behaviors for different bioactives at different incubation periods and concentrations. Among the bioactives tested, LOB-A showed a reverse concentration-response with extended incubation periods of 48 hours and reduced protection of the epithelial barrier for the highest concentration at 24 hours (200 nM). Prior to these experiments we evaluated the cytotoxicity of all bioactives to define our working concentrations, and LOB-A showed potent cytotoxicity at lower concentrations when compared to ENL and other LOBs. Nonetheless, we used this to aid treatment concentrations, but higher concentrations of LOB-A might have induced apoptosis with enhanced loss of barrier integrity in the *in vitro* system. This can be explained by increased stress on cells in transwells, and the use of Hanks Balanced Saline Solution (HBSS) for an extended duration making cells more fragile and susceptible to cytotoxicity at lower concentrations of LOB-A. Unlike LOB-A, ENL showed a mild protection at 24 hours and a more consistent concentration-response effect at 48 hours. This observation could potentially be attributed to what we observed with enhanced effects after conjugation with glucuronide (Chapter 5), where forty eight hours duration is sufficient for intestinal epithelial cell lines to introduce a glucuronide moiety to ENL[219,221]. Typically, glucuronidation is considered a detoxification pathway resulting in pharmacologically inert metabolites. In some instances, this metabolic pathway results in an active metabolite and in some cases a very potent drug (e.g. morphine-6-glucuronide, morphine-3-glucuronide, ezetimibe-glucuronide)[352]. Similarly, we speculate that ENL has to undergo glucuronic acid conjugation to provide anti-inflammatory and cholesterol reduction effects.

To further evaluate the anti-inflammatory effect, we assessed ENL and LOBs effects on the genetic expression of several cytokines, chemokines and inflammatory mediators. Interestingly, ENL and LOB-A caused upregulation of PPAR-γ. This transcription factor is involved in several

critical metabolic and immune functions and contributes to important side effects in the human body[373]. In IBD, PPAR-γ downregulation is associated with IBD in murine models and UC in humans[265, 266]. In addition, the PPAR-γ agonist, rosiglitazone[269, 374], is clinically proven to effectively treat mild to moderate UC in humans[270], suggesting its critical role in IBD. In a related fashion, our data suggests that the increased expression of PPAR-γ associated with ENL and LOB-A will positively reduce inflammation in IBD in general and with higher odds of benefits in UC patients.

Oxidative stress is a hallmark of IBD pathogenesis, where increased reactive oxygen species are reported to alter barrier integrity and exacerbate immune reactions[146, 151]. In addition to assessments of barrier integrity, we used a co-culture system that closely simulated the intestinal epithelium environment that is also capable of producing significant amounts of reactive oxygen species. In this system, the Caco-2 cell line was plated on the apical side of the transwell and RAW 264.7 macrophage cell line was introduced to the basal compartment[276]. Due to abundant expression of Toll-Like receptors in RAW 264.7, inflammatory induction was carried out with LPS rather than with TNF-α and INF-γ[375]. In assessing intracellular oxidation experiments using the fluorogenic DCFH-DA oxidation marker, ENL and LOBs reduced fluorescence (as an indicator of oxidative stress) with the exception of LOB-J. When evaluating secondary oxidative stress protection through enzymatic modulation, ENL was superior and consistent relative to the LOBs. Most importantly, when we assessed the effect on ROS-damaged lipids through an MDA index of lipid peroxidation we found that ENL and LOBs provided a protective effect. Therefore, we hypothesize that ENL consistency and superiority in ROS suppression could be attributed to its capacity to act as both a ROS scavenging agent by the virtue of its phenolic structure, and through transcriptional and enzymatic modulation due to its small size (298.3 g/mol) and capacity to permeate through the cell membrane. This role can also be supported by evidence of PPAR-γ upregulation (Chapter 2), which is also suggested to be involved in cellular protection against oxidative stress[336, 376], confirming involvement of intracellular transcriptional modulation. But it is unlikely that LOBs are involved in transcriptional and intracellular processes, due to their high molecular weights and unlikely ability to permeate the cell membrane thereby mostly acting through neutralization of free radicals by activity at the extracellular cell membrane surface or by other cell surface mechanisms[377, 378].

Barrier integrity in the co-culture model of the Caco-2 intestinal cell line and RAW265.7 macrophage cell line confirmed previous results observed in the HCT-8 intestinal cell monoculture system. Enterolactone showed a concentration-response effect that was maintained for up to 48 hours, which relates again to the possible role of the glucuronidated metabolite rather than unconjugated ENL. Unlike ENL, the concentration-response behavior of LOBs was lost in some instances, which could indicate a lack of stability for those molecules in an *in vitro* system. LOB-A and LOB-J are stable and are not subject to chemical oxidation. For LOB-A at high concentration (2 µM) the cellular stress caused by the combination of LPS and incubation in HBSS media might result in increased sensitivity of the cells to this LOB resulting in toxicity and cell death of RAW 265.7, which is the source of inflammation. For LOB-J, the lowest concentration (20 nM) employed was most effective, but this protection was lost with higher (high nM and µM) concentrations after eight hours. This possibly suggests a clear immunosuppressive behavior. Interestingly, when combined together LOB-ACEJ was most effective at the mid- concentration (200 nM) possibly supporting the notion that higher concentrations of LOB-J counteract the benefits of high LOB-A concentrations. Despite the inconsistent behavior of LOBs, both ENL and LOBs impart a significant barrier protection on Caco-2 polarized epithelium in a co-culture system.

In addition to barrier integrity and previous experimentation on genetic expression changes in the HCT-8 cell line, we opted to investigate the effect of ENL and LOBs on innate and adaptive immune response through a dedicated microarray. For this purpose, we used the RAW 264.7 cell line and pooled concentrations of ENL and LOBs. The selection of the RAW 264.7 cell line (macrophages) was made because of their role in both acute and chronic inflammatory response reported in IBD pathogenesis. In addition, the RAW 264.7 cell line is susceptible to LPS sensitization as an alternative to microorganism infection. Furthermore, RAW 264.7 and Caco-2 utilized the same growth media with plausible growth conditions that renders *in vitro* simulation reasonably performable. Results from the microarray experiment identified Interleukin-5 (IL-5) as a major player involved in the immune response induced by pooled ENL and LOBs. The major role of IL-5 is reported to be in eosinophilic activation, and controversy exists regarding its beneficial/harmful role in IBD. Eosinophil recruitment is involved in clearing parasitic, viral and bacterial infection[289-291], making it possible to help clear infections before exacerbation of intestinal inflammation[288]. Recently, a shift in paradigm has been suggested by studies showing

that worm infections (that activate IL-5) are associated with selective microbiotal colonization that favours protection of barrier integrity[379]. Interestingly, in the DSS colitis murine model, activation of the eosinophilic pathway was associated with attenuation of gastrointestinal inflammation[292].

To further understand the role of ENL and LOBs in immunological processes, we utilized microarray data obtained from pooled ENL and LOBs treatment in RAW 264.7 cells in gene set enrichment analysis (GSEA). We further used a set of 20 genes (top 20 down-regulated genes) and used gene set enrichment for those genes in a human metabolome database utilizing Expression2Kinases software[380]. This method provides information on how a list of genes correlate to a set of terms in a specific database, and further ranks these correlations by their significance. In our case, the top 20 downregulated genes that were generated from microarray data analysis were applied into Expression2Kinases software. This evaluation resulted in a significant enrichment of terms in table 6.1, including phenylalanine, atorvastatin, formaldehyde, hydrogen peroxide, heme and iron. Both, L-phenylalanine and atorvastatin are of critical interest to our hypothesis. The latter (atorvastatin) is a blockbuster agent used to treat variants of hypercholesterolemia, and L-phenylalanine is a potent inhibitor of pyruvate kinase M2 (PKM2)[381, 382]. These two terms suggest the involvement of ENL and LOBs in both pharmacological functions our hypotheses are trying to test. The relationship to our hypotheses comes as atorvastatin is directly involved in cholesterol metabolism and modulation, and PKM2 acts as a key bridge between cellular energy consumption and inflammation[320]. Therefore, we decided to further investigate the utility of PKM2 as a potential biomarker in IBD. Our study involved extracting information from a previous newly diagnosed IBD patient dataset on IBD patient diagnosis, fecal IBD biomarker values, and microbiota content and correlating these to serum PKM2 concentrations utilizing ELISA for quantification. Our assessment of serum PKM2 suitability as a possible biomarker shows that it is correlated with IBD in general but cannot differentiate between CD and UC. The use of serum PKM2 may explain the inability to differentiate the IBD patient data set and we surmise that fecal PKM2 may have more relevance, due to our understanding of PKM2 being an abundant protein in epithelial tissues and other highly proliferative cell types such as neoplastic cells[304, 305]. In addition, the difference between CD and UC is also correlated to the length of damage in the intestinal epithelium structure, where UC is extended over the length of the colon and CD tends to be patchier and localized. Therefore, we predict that PKM2 fecal levels might show superior correspondence to UC than CD, if assessed.

Table 6.1. Gene set enrichment analysis (GSEA) of top 20 down-regulated genes in respect to human metabolome database. GSEA was performed using freely available software (Expression2Kinases).

Term	Molecule
HMDB00159	L-Phenylalanine
HMDB05006	Atorvastatin
HMDB01426	Formaldehyde
HMDB03125	Hydrogen peroxide
HMDB03178	Heme
HMDB00692	Iron
HMDB00547	Magnesium

Concurrent experimentation for the discovery of a biomarker in IBD by Marc Morris facilitated these experiments through microbiota, serum and other biomarkers quantification[383]. Marc reports that the inclusion of several biomarkers in scoring models will improve the predictability of disease relapse and response to therapy clinically when compared to patient reported outcomes. In concordance with this we see that our experiments together might further the development of a panel of biomarkers that will fortify the selectivity, specificity and utility of serum and fecal biomarkers in the clinical setting.

In addition to our GSEA that listed atorvastatin as a significantly enriched term in the downregulated gene set, the literature reports lignan involvement in cholesterol modulation and metabolism. In our studies, we developed a novel method for assessing the effect of ENL and LOBs on cholesterol absorption. Our initial assumption that lignans (ENL) inhibit cholesterol absorption was due to lack of evidence supporting an effect on cholesterol metabolic enzymes and potential similarity to sesamin lignan[335]. This investigation was challenging due to the lack of *in vitro* methods that differentiate between a cholesterol absorption inhibitor and cholesterol metabolism modulator. The use of U18666A, a cholesterol trafficking molecule that traps absorbed cholesterol intracellularly, helped eliminate the possibility of lignan effect on cholesterol uptake,

due to the persistent appearance of fluorescing cholesterol (NBD-cholesterol) after co-treatment with ENL and ENL-Gluc. The absence of NBD-cholesterol upon use of the approved cholesterol absorption inhibitor Ezetimibe-Gluc supported the idea that the lignans fail to influence cholesterol uptake.

Clinically, a hypocholesterolemic effect is observed with oral supplementation with lignans from flaxseed and other sources[82, 84, 384]. The mechanism through which lignans modulate cholesterol is still unknown. Our results showed that ENL-Gluc upregulated INSIG-1 expression. To elaborate more on INSIG-1 functions, under cholesterol rich conditions, INISIG-1 acts as an anchor protein to trap sterol regulatory element-binding proteins and its cleavage-activating protein (SREBP-SCAP) in the endoplasmic reticulum and prevent their dissociation and activation of cholesterol synthesis[356, 357]. In addition, INSIG-1 upregulation increases the sensitivity of cells to sterol-mediated feedback inhibition of cholesterol synthesis[360], suggesting fortified activity at increased levels of cellular cholesterol. This is consistent with clinical reports that showed a potential beneficial effect in hypercholesterolemic patients[11, 82], but not in the healthy population [98].

In alignment with INSIG-1 upregulation, the observed upregulation of PPAR-γ that we have seen as an anti-inflammatory indicator is intertwined with cholesterol metabolism. König *et al.*[384] provided evidence on how fibrate- and thiazolidinedione-mediated upregulation of PPAR-γ will upregulate INSIG-1, -2 and downregulate SREBP-1, and eventually reduce *de novo* synthesis of cholesterol. Therefore, the dual activity of upregulating PPAR-γ which will result in increased barrier integrity and anti-inflammatory status, in addition to upregulation of INSIG-1 that will reduce cholesterol synthesis, supports our hypothesis of flaxseed involvement in IBD and hypercholesterolemia. Furthermore, this adds more reliability of utilizing PKM2 as a biomarker for IBD as both energy generation and immune response is becoming more interconnected.

This research provides important information about how different bioactives from flaxseed might trigger different anti-inflammatory mechanisms. In addition, this work identifies the potential need for ENL glucuronide conjugation to observe the beneficial hypocholesterolemic effect of flaxseed lignan consumption, which provides important information to translate this research into animal models. Therefore, these studies were premised on evaluating key active components of flaxseed to ultimately provide more robust *in vitro* (and subsequently animal

model) evidence for their ability to ameliorate inflammation and modulate cholesterol metabolism, to utilize in pathologies like IBD and hypercholesterolemia.

6.2 Challenges and limitations

This research faced several challenges. At the beginning of the project the main challenge was the induction of inflammation and disruption of barrier integrity in a transwell system that is composed of polarized intestinal epithelium cells. Upon induction with either TNF-α or LPS, reduction in TEER was not observed. This was due to lack of complete protocols for *in vitro* inflammatory induction guidelines, where small details impart a significant difference. For example, excess glutamate in media has a significant protective effect on barrier integrity. In addition, significant inter-lab variation existed with respect to the concentration of the inflammatory stimulus used. Therefore, several optimization studies had to be performed. Similarly, the duration of incubation in the transwell system to ensure a polarized epithelium with high integrity varies among labs, and in house optimization was a necessity. This *in vitro* design has its limitations as utilization of Caco-2 and HCT-8 cell lines will simulate and correspond to different specific areas of the gastrointestinal system. Given our understanding of the gastrointestinal system where different regions impart different physiological functions, one cell line system will not duly simulate the whole organ. Further, the co-culture system where we used RAW 264.7 cell (mouse macrophages) is limited by the potential cross-species immunogenic reaction. Later, when we started assessing LOBs effects, the erratic behavior and lack of concentration-response relationships in some experiments caused some concern on the stability of these compounds *in vitro.* Our experiments were limited by the number of bioactives we tested and the need to select a limited number, which excludes valuable information concerning the potential of other bioactives in favourably modulating cholesterol homeostasis and the hallmarks of IBD pathogenesis. Another limitation is that the LOB combinations (e.g. LOB-ACEJ) we used to understand their pharmacological activity cannot fully represent the actual ingestion of a standardized natural product. This is due to the lack of understanding of ratios of each individual component and how accurate is its representation in a standardized natural product.

In the evaluation of PKM2 levels in newly diagnosed IBD patients, the main challenge was the need for greater patient recruitment to increase the sample size to enhance the robustness of PKM2 utility as a biomarker. Another challenge that will remain is to develop a protein

quantitation method that encompasses all the forms of PKM2 (dimeric, tetrameric and monomeric). These forms have different levels of endogenous activity, where the tetrameric form has the highest metabolic activity and dimeric form has the least[302]. Therefore, a simple straight forward method to selectively analyze all these at once may enhance clinical utility and allow stronger correlations.

6.3 Future work

This current research is helpful to establish several other future work under the umbrella of translational health research for natural products. Due to the variable techniques and pathologies examined in this research, different directions in my future work will take place.

In regards to the lack of the concentration-response relationship for LOBs in some instances, stability studies for those LOBs is a necessity. Despite the availability of industrial scale stability, we propose that a systematic analysis of stability in a drug discovery unit is critical for proceeding with using LOBs as potential anti-inflammatory mediators. This can be done through the development of an analytical method and accurate quantitation by LC-MS/MS. In addition, to confirm that LOBs will lack permeation through the intestinal epithelium and exert a local effect, we suggest extensive permeability studies be performed to further justify the utilization as a local anti-inflammatory in the intestine.

After confirmation of stability in different *in vitro* medias, pharmacological activity should be translated to appropriate animal models that are consistent with results in this dissertation research, specifically in regards to INSIG-1 upregulation. This model should utilize animals that are already hypercholesterolemic which are suitable for the pathways in which INSIG-1 is involved, this could include diet-induced hypercholesterolemia due to what has been observed in human populations. Subsequently, after induction of hypercholesterolemia, administration of a standardized lignan enriched flaxseed product for a period of time would be followed by blood collection to assess lipid profile (e.g. total cholesterol, triglycerides, LDL-cholesterol and HDL-cholesterol), in addition to physical measures (e.g. central obesity), histopathological assessment of lipid tissues and blood vessels, and molecular and biochemical evaluations to confirm INSIG-1 pathway involvement *in vivo*.

Finally, the identification of PKM2 as a biomarker in IBD needs to be expanded to include more patients to improve the robustness of PKM2 as a biomarker. Furthermore, elevated PKM2 should be verified with samples obtained from participants' feces, where fecal samples might

improve the differentiation between CD and UC due to differences in the intestinal surface involved in inflammation between the two pathologies. This comes in the alignment of PKM2`s extended involvement in epithelial cells. Furthermore, assessment of all isoforms of PKM2 must be carried out for possible identification of more selective testing kits. These results, in addition to what have been reported by our colleagues about the usage of the composite scoring systems, might incite the development of a biomarkers panel that encompasses PKM2 to help diagnose and stratify the nature of disease activity.

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